Investigation of the effects of hyaluronan on human monocytes and macrophages in inflammatory processes.

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For Dad
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Declaration

I declare that this thesis was written by myself, and that all experiments were performed by myself.

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Abbreviations

ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid
ARDS	Adult respiratory distress syndrome
BAL	Bronchoalveolar lavage
bFGF	Basic fibroblast growth factor
Bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CF	Cystic fibrosis
CFA	Cryptogenic fibrosing alveolitis
Da	Daltons
DAB	3,3' Diaminobenzidine
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hyaluronan
HaBR	Hyaluronan binding protein
HBSS	Hanks-buffered salt solution
HRP	Horseradish peroxidase
ICAM-1 (CD54)	Intracellular adhesion molecule 1
ICE	Interleukin-1β converting enzyme
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
kb	Kilobases
kDa	Kilodaltons
LIF	Leukaemia-inhibitory factor
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex

MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein-1α
ONPG	o-nitrophenyl β-D-galactopyranoside
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKC	Protein kinase-C
PMA	Phorbol myristate acetate
PRP	Platelet-rich plasma
PPP	Platelet-poor plasma
PXB	Polymyxin B
RANTES	Regulated on activation, normal T cell expressed and secreted
RDS	Respiratory distress syndrome
RHAMM	Receptor for hyaluronan-mediated motility
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecyl sulphate
TACE	Tumour necrosis factor-alpha converting enzyme
TBS	Tris buffered saline
TEMED	N,N,N'N'-tetramethylethylenediamine
TGF	Transforming growth factor
TNFα	Tumour necrosis factor-α
TMB	3,3'5,5'-Tetramethylbenzidine

Abstract

Cells of the monocyte-macrophage lineage play a central role in inflammation and in the pathogenesis of inflammatory disease states. During inflammatory responses, these cells are able to recognise and eliminate invading micro-organisms via efficient phagocytic and cytocidal host defence mechanisms. In addition to this effector role, monocytes and macrophages can produce a vast array of cytokines and chemokines which affect the responses of other cells and can influence the progression of inflammation.

From the moment of extravasation into areas of inflammation, the surrounding microenvironment has a profound effect on monocytes and macrophages and can influence the differentiation process that occurs in monocytes within different tissues, or at different stages of inflammation.

During extravasation, monocytes and macrophages encounter elements of the extracellular environment, normally arranged in a stable structure of proteins, glycoproteins, and proteoglycans, known as the extracellular matrix. At sites of injury, this stable structure is simulated by a provisional matrix, which is formed as a result of vascular coagulation, and is one of the first influences on elicited cells as they migrate into the inflammatory site.

This study shows that hyaluronan, which is a ubiquitous component of the extracellular matrix, and a major component of the provisional matrix , has a pro-inflammatory effect on monocytes and macrophages. This effect was not observed with other ECM components, including fibronectin, vitronectin, collagen I and heparan sulphate, which is structurally related to hyaluronan.

In this thesis, the pro-inflammatory effects on both monocytes and monocyte-derived macrophages were examined. The effects of hyaluronan on monocytes were found to include the production of cytokines, specifically TNFα and IL-1β. Monocyte-derived macrophages also responded to hyaluronan with the production of TNFα. This response involved the hyaluronan receptor CD44, the expression of which is increased upon *in vitro* maturation.

These pro-inflammatory effects of hyaluronan may have serious implications for the pathogenesis of many inflammatory disease states. The adult respiratory distress syndrome is an example of an acute inflammatory state which can progress to a chronic and maladaptive inflammatory response. Levels of hyaluronan in the bronchoalveolar lavage fluid from patients with ARDS were measured by ELISA, and were found to be significantly elevated. The presence of hyaluronan was also revealed in the alveolar interstitium of lung sections taken from patients with ARDS. Elevated hyaluronan associated with ARDS may result in the escalation of inflammatory responses. As the main immunocompetent cell in the lung is the alveolar macrophage, the pro-inflammatory influence of hyaluronan on macrophage responses may be instrumental in the pathogenesis and progression of disease. Together the results presented in this thesis suggest a pivotal role for hyaluronan in the regulation of monocyte-macrophage inflammatory responses.

Chapter 1

1. Introduction

1.1 The inflammatory environment

Inflammation is an important consequence of tissue injuries caused by various stimuli such as physical trauma, bacterial and viral infections, xenobiotics and autoimmune diseases. Acutely, inflammation is characterised by vasodilation, increased vascular permeability, and cellular infiltration, a series of cellular and molecular events, which act in concert to minimise tissue damage and eliminate the provoking stimuli.

The inflammatory response is initiated through the vascular coagulation mechanism, which is an overlapping and sequential series of events. A complex system of circulating pro-enzymes, cofactors, and inhibitors leads to the activation of platelets and the formation of a fibrin-platelet clot at the site of vascular injury. The deposition of plasma fibrinogen, its conversion to insoluble fibrin and further cross-linking with other fibrin molecules reinforces the platelet plug and forms a provisional matrix, which provides a physical barrier to the spread of infection (Brown et al., 1988). Thrombin release associated with coagulation leads to platelet degranulation, which results in the release of chemotactic and growth promoting cytokines at the inflammatory site (Raines and Ross 1982, Assoian and Sporn 1986, Weyrich et al., 1996). Thus inflammation is delimited by the provisional matrix, yet persists locally until the circumstances responsible for its initiation are resolved.

Control of inflammatory processes is ensured through local activation or release of sequestered mediators at the inflammatory site, which initiate cellular recruitment

and the process of repair. Vasoactive chemicals such as histamine (from mast cells and basophils), prostaglandins (from polymorphonuclear cells, monocytes and platelets) and leukotrienes (from neutrophils, mast cells and basophils) are produced which rapidly increase vascular permeability and cause vasodilation. The increased vascular permeability results in oedema, due to the leakage of plasma across the endothelium, this inflammatory exudate ultimately drains to lymph nodes, where initiation of antigen-specific immune responses may occur. Vasodilation provides conditions that favour maximal infiltration of inflammatory cells (Busse 1979, Pecke and Williams 1977, Bray et al., 1981).

Neutrophils are the first infiltrating cells in acute inflammation and arrive within minutes (Hurley et al., 1966). These highly phagocytic granulocytes are capable of releasing a large number of histotoxic agents through degranulation (Haslett et al., 1989), providing an effective defence against the spread of infection. During the progression of inflammation the numbers of monocytes and lymphocytes gradually increase (Issekutz et al., 1981), these remove remaining debris and initiate repair. Removal of granulocytes from the inflammatory site during the resolution of inflammation is thought to involve apoptosis, followed by phagocytosis by macrophages and other phagocytes (Savill et al., 1989). Cells undergoing apoptosis are characterised by a series of morphological and phenotypical changes (Wyllie et al., 1980, Savill et al., 1989, Whyte et al., 1993, Dransfield et al., 1994) which serve to functionally isolate the cells from potential pro-inflammatory stimuli and allows them to be recognised and phagocytosed by macrophages. In contrast the fate of the macrophage at inflamed sites is less well defined. Although monocytes and macrophages are capable of undergoing apoptosis *in vitro* (Mangan et al., 1991,

Munn et al., 1995) macrophages *in vivo*, appear to leave the inflammatory site via the lymphatics (Lan et al., 1993, Bellingan et al., 1996). In the lung, macrophages may also be transported out by the mucociliary escalator (Weibel 1984).

Throughout the inflammatory process macrophages continue debridement through active phagocytosis, and initiate the transition from inflammation to repair through the secretion of growth factors that promote angiogenesis (Polverini et al., 1977) which is essential for neovascularisation and connective tissue formation. The recruitment of fibroblasts results in the laying down of various extracellular matrix molecules (Ross et al., 1970), thereby promoting the formation of granulation tissue. After considerable reorganisation, granulation tissue gradually disappears and is replaced by the more organised and elastic extracellular matrix, which forms scar tissue (Clarke 1990).

Reintroduction of, or failure to remove the inflammatory stimulus may lead to conditions in which inflammation becomes chronic. Tissue macrophages are principal regulators of inflammatory processes through the production of various inflammatory mediators, including the cytokines interleukin-1, interleukin-8, and tumour necrosis factor-α. The possibility that altered patterns of cytokine production by macrophages may shift the repair process towards a chronic inflammatory state is examined further below.

1.2 Macrophage Origin

Monocytes, which make up 2-8% of peripheral blood leukocytes, are formed in the bone marrow from progenitor cells (reviewed by Dorshkind 1990). Differentiation from pluripotent progenitor cells is controlled by at least 11 different growth factors, with IL-3 regulating the progression to myeloid restricted progenitors. These cells then become responsive to M-CSF and GM-CSF which are responsible for their maturation to monocyte/macrophage-restricted progeny (Rutherford et al., 1993). Following release from the bone marrow, monocytes circulate in the bloodstream for less than 72 hours prior to entering tissues (extravasation)(van Furth and Cohn 1968, Volkman 1970, Whitelaw and Batho 1972) and can be considered to function as a circulating pool for constitutive and induced recruitment. In the absence of inflammatory stimuli, recruited monocytes may differentiate into resident macrophages, with distinct phenotypes in different organs illustrating their potential functional diversity.

There is some evidence that suggests that tissue macrophage populations may be maintained by proliferation within tissues. Although monocyte precursor cells are not produced to any great extent outside the bone marrow (van Furth and Cohn 1968), van Furth and Diesselhof-den Dulk (1984) showed that the spleen in mice retains some haematopoietic activity and therefore a capacity for macrophage production. Volkman et al., (1983) suggested that some proliferation may occur in the lung, liver and peritoneal cavity, although the extent to which this occurs is not certain.

The phenotypic heterogeneity of macrophages has complicated the analysis of their distribution within tissues. However, it is now well established that macrophages are present throughout the connective tissues and around the basement membrane of small blood vessels. They are particularly concentrated in the lung, liver, and lining of spleen sinusoids and lymph node medullary sinuses, consistent with their proposed role as scavenger cells in innate immunity. They are also capable of differentiating into brain microglia, and osteoclasts in bone (Gordon 1995). Thus the differentiation of macrophages is essential for their functional competence within different tissues.

A variety of environmental signals in situ modulate monocyte differentiation to macrophages. Circulating monocytes extravasate in response to a chemotactic gradient, requiring adherence to the vascular endothelium and migration involving receptor-mediated transient cell-cell and cell-extracellular matrix adhesive interactions. These adhesions are mediated through cell surface receptors and represent the initial influence in monocyte to macrophage maturation.

Many adhesive surface molecules on monocytes have been described, which on the basis of their biochemical properties can be grouped into three main families of receptors; the immunoglobulin (Ig) supergene family, the selectins, and the integrins.

The Ig family members are composed of a number of extracellular Ig-like domains arranged in tandem, which are either glycosylphosphatidylinositol (GPI)-linked e.g., CD16, or have a transmembrane and a cytoplasmic domain (Williams and Barclay 1988). Examples include intercellular adhesion molecules 1, 2, and 3, VCAM-1, and CD31 (reviewed by Springer 1990).

The selectins comprise CD62E (E-selectin), CD62L (L-selectin), and CD62P (P-selectin), and at a structural level contain an NH_2-terminal lectin domain, an EGF-like domain, consensus repeat sequences, a transmembrane domain, and a cytoplasmic domain (Varki 1997). These molecules are involved in the loose tethering of leukocytes to the vascular endothelium (Lawrence and Springer 1991). The selectins are perfect for this role due to their molecular structure which extends the lectin domain above the surrounding glycocalyx and allows them to sequestrate passing leukocytes expressing the appropriate receptors (Lasky 1992). In addition to a role in regulating the production of cytokines (Weyrich et al., 1995), adhesions mediated by selectins allow the leukocyte to roll along the vessel wall "searching" the endothelial surface for the presence of activating cytokines. Where such factors are absent the transient nature of the selectin mediated adhesion allows the leukocyte to continue rolling or to disengage and rejoin the circulation. In contrast, inflammatory cytokine actions on the leukocyte convert functionally inactive integrin molecules to an adhesive configuration (Detmers et al., 1990, Vaddi and Newton 1994, Weber et al., 1996). The integrins, so called because of their integral membrane nature and role in the integrity of both the ECM and cytoskeleton (Tamkun et al., 1986), are made up of an α-chain non-covalently linked to a β-chain and provide strong adhesion to endothelium (Hynes 1987).

Beside these three main families, a variety of other molecular structures have been shown to have a role in adhesion processes, including CD44 a molecule which was first identified as a putative "homing" receptor for lymphocytes (Goldstein et al., 1989, Stamenkovic et al., 1989). Several functions have been attributed to CD44,

including binding to high endothelial venules (Jalkanen et al., 1988) binding to collagen and fibronectin (Carter and Wayner 1988, Jalkanen and Jalkanen 1992) and binding to hyaluronan (Aruffo et al., 1990, Culty et al., 1990, Miyake et al., 1990).

Consistent with the suggestion that monocyte adhesion and the immediate micro-environment provides signals which contribute to macrophage differentiation, Henson and Riches (1994) proposed a number of maturation pathways in the mouse resulting in functionally and phenotypically distinct macrophages. One such pathway, induced by CD44 ligation with hyaluronan and the additional influence of TNFα gave rise to an IGF-1 synthesising macrophage which might be considered pro-fibrotic in function. Cytocidal macrophages, secreting reactive nitrogen intermediates and complement factor b, may be derived from the combined influences of interferons and TNFα on lipopolysaccharide-primed monocytes. A more inflammatory macrophage, expressing lysosomal enzymes, is derived (*in vitro*) through stimulation with β1,3-glucan (the principal constituent of yeast cell walls). These data support the suggestion that there may be several routes for the differentiation of monocytes to macrophages, resulting in functionally competent cells adapted for specific microenvironments, in both inflammatory and steady state conditions.

1.3 Macrophage Function

Macrophages play a central role in the maintenance of homeostasis in the immune system, and are thought to be a key cell type the pathogenesis of many disease processes.

1.3.1 Macrophages as phagocytes

Macrophages are efficient phagocytes with a role in inflammatory processes, identified as such at the turn of the century by Metchnikoff (1884). Phagocytosis can be considered to involve 3 stages; recognition and binding, internalisation, and digestion. Cell membranes carry a negative charge that keeps them apart and prevents autophagocytosis, and the hydrophobic nature of the lipid bilayer prevents hydrophilic bacterial cell wall components from entering the cell. To overcome these difficulties, phagocytes have specific cell surface receptors that mediate the attachment of opsonised particles. Macrophages have 2 receptors for IgA (FcαR) (Maliszewski et al., 1990), and 3 types of receptors which bind to the Fc portion of immunoglobulin G isotypes (reviewed by van de Winkel and Capel 1993). These are FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), FcγRI has a high affinity for human monomeric IgG of all isotypes, whereas FcγRII and FcγRIII are low affinity receptors, and bind aggregated IgG (Corvaia et al., 1995). Fc receptors are also expressed on monocytes, and their ligation can induce monocyte activation (McIntyre et al., 1989). One feature of monocyte to macrophage maturation is the altered expression of Fc receptors (Clarkson and Ory 1988). Opsonisation of particles by complement components may result in the binding to surface receptors including CD35 (complement receptor 1 or CR1) which binds C3b and C3dg, and the β2 integrin CD11b/CD18 and CD11c/CD18 which recognise iC3b (Wright et al., 1983, Malhotra et al., 1986). Non-opsonised particles may be phagocytosed through the co-operative action of different receptors, in particular those recognising carbohydrate moieties. For example, the macrophage mannose receptor which

mediates the binding and ingestion of microorganisms with surface mannose residues and soluble mannose-containing glycoproteins (Ezekowitz et al., 1990, Stein et al., 1992). Alternative receptors capable of promoting opsonin independent phagocytosis include β2 integrin (CD11b/CD18) (Greenberg 1995), scavenger receptors (Krieger and Herz 1994) and β1 integrins, even though these receptors generally do not mediate efficient phagocytosis in the absence of ligand binding (Isberg and Tran van Nhieu 1994). Receptor ligation leads to the particle becoming engulfed, and destroyed by a variety of enzymes including lysosyme, and reactive oxygen or nitrogen intermediates. Resident macrophages in different organs display phagocytic functions to varying degrees, which may reflect the appropriateness for that particular micro-environment. For example, alveolar macrophages are highly phagocytic, possessing high levels of the mannose receptor, in contrast epidermal macrophages (Langerhans cells) are poorly phagocytic but are highly efficient antigen presenting cells.

1.3.2 Antigen presentation

Macrophages and dendritic cells are considered to be the classical antigen presenting cells within lymphoid organs but macrophages are the primary antigen presenting phagocytic cells. All nucleated cells express MHC class I molecules which are involved in the presentation of endogenous antigen but MHC class II molecules are involved in the presentation of exogenous and endogenous antigen. MHC Class II molecules, which are heterodimers (composed of a 33-35kDa α chain and a 25-

29kDa β chain) are also expressed on B-cells and activated T-cells (Brodsky and Guagliardi, 1991).

T-cells are the effector cells in antigen responses and because the T-cell antigen receptor does not recognise free antigen, macrophages must "present" antigen in a form easily recognised. Antigen presentation involves the association of peptide fragments derived from the antigenic proteins, with MHC class II molecules (reviewed by Brodsky and Guagliardi 1991). Antigen that enters the tissues is transported through lymph to the draining lymph node, either by antigen presenting cells or free in the exudate. MHC Class II molecules assemble in the endoplasmic reticulum with the invariant chain to form a stable trimer (reviewed by Sant and Millar 1994). The invariant chain is a 31-43kDa glycoprotein which is thought to inhibit the binding of peptides to MHC Class II in the endoplasmic reticulum (Braciale and Braciale 1991). The invariant chain is proteolytically cleaved from the MHC Class II molecule binding cleft in the endosome to allow degraded antigen to bind and be transported to the cell surface where it can be presented to T-cells.

1.3.3 Secretion

In addition to their roles in phagocytosis and endocytosis, macrophages are capable of secreting a variety of small and macromolecular products that mediate immune responses, ranging in molecular mass from 30 (nitric oxide) to 440000 (fibronectin) (Laskin and Pendino 1995). Other secreted products include cytotoxic agents such as reactive oxygen and nitrogen intermediates, proteases, and lysosomal enzymes released during the phagocytic response. Macrophages are also capable of producing

a variety of growth factors and extracellular matrix proteins. Cytokine secretion, in response to inflammatory agents, microbial invasion and tissue injury can contribute to the resolution of inflammation and restoration of normal homeostasis. However over- or underproduction of certain cytokines can contribute to pathogenesis of diseases such as endotoxaemia and the acute respiratory distress syndrome (ARDS).

1.4 Macrophage Cytokines

Cytokines are usually low molecular weight glycoproteins that are produced by various cell types and can act in an autocrine or paracrine manner. Cytokines often induce synthesis of, and affect actions of other cytokines resulting in a complex network. The macrophage plays a pivotal role in the cytokine network, producing various pro-inflammatory cytokines (including TNFα, interleukin-1, interleukin-6, and interferons), chemotactic cytokines (e.g., interleukin-8 and monocyte chemotactic protein-1), and pro-fibrotic cytokines (e.g., transforming growth factor-β) see table 1.

TNFα was first reported in 1975 as a protein in sera that caused tumour regression when injected in mice (Carswell et al., 1975) and is responsible for the features of septic shock once attributed to endotoxin (Beutler and Cerami 1986). It is expressed as a 26kDa membrane-bound precursor containing a hydrophobic sequence that is proteolytically cleaved by TACE (Black et al., 1997, Moss et al., 1997) to form the mature 17kDa polypeptide. In solution, mature TNFα exists as a compact non-

covalently linked trimer of 52kDa (Fiers 1993). Synthesis of TNFα is stimulated by numerous agents including bacterial cell wall derived lipopolysaccharide (LPS), fungal products, and viral particles. Like many cytokines, expression of TNFα is controlled at the level of gene transcription and translation. Although unstimulated monocytes express low levels of TNFα mRNA, upon stimulation upregulation of both translation and transcription of the gene product occurs within minutes (Beutler et al., 1986). In murine macrophages TNFα signalling has been shown to involve $NF_{\kappa}B$ (Shakov et al., 1990), however the involvement of $NF_{\kappa}B$ in the transcriptional activation of TNFα in human monocytes is more controversial. Goldfield et al., (1990) failed to show $NF_{\kappa}B$ site involvement in the transcriptional activation of human TNFα using murine monocytic cells, although TNFα has been suppressed by inhibitors of $NF_{\kappa}B$ nuclear translocation in monocytic cell lines since then (Zeigler Heitbrock et al., 1993, Oeth and Mackman 1995). The data on TNFα signalling pathways in human peripheral blood monocytes or human macrophages is lacking.

There are two distinct receptor sub-types for TNFα, designated p55 (55kDa) and p75 (75kDa) (Hohmann et al., 1990, Brockhaus et al., 1990). Within minutes of binding, the receptor-ligand complex is internalised and before being degraded, the intracellular signal is transmitted by mechanisms not yet elucidated. However, the response of the cell correlates with the number of complexes internalised, thus shedding of these receptors may provide a mechanism for down modulation of TNFα activity. TNFα induces a number of proteins through the activation of transcription; the induction of CD62E on endothelium is achieved through $NF_{\kappa}B$ (Read et al 1997), as is the activation of neutrophils (Macdonald et al., 1997).

Interleukin-1 has also been shown to be a pluripotent mediator in the pathogenesis of inflammation. Although IL-1 does not appear to induce irreversible tissue damage alone, it acts to potentiate many of the effects of TNFα. IL-1 and TNFα both increase the adhesiveness of endothelium for leukocytes via the upregulation of CD62E and CD62P expression, integrin activation and induction of immunoglobulin superfamily members ICAM-1 and VCAM-1 (reviewed by Beekhuiken and van Furth 1993). Both cytokines have been shown to induce an aggressive matrix degradative phenotype in fibroblasts (Pettipher 1986). The membrane bound form of IL-1 is cleaved by a calcium dependent cysteine protease (interleukin-1B converting enzyme or ICE) which has been identified and cDNA cloned (Thornberry 1992).

TNFα and IL-1 are released early in inflammation whereas other cytokines e.g., interleukin-6 and interleukin-8 are released later. Such evidence suggests that inflammatory stimuli initiate a cytokine cascade sequentially involving TNFα interleukin-1, interleukin-6, and interleukin-8. This suggestion is supported by analysis of cytokine production in studies of disease progression in the inflammatory disorder rheumatoid arthritis (Smolen et al., 1996).

Table 1. Principal macrophage cytokines and functions.

Cytokine	Molecular Weight (kDa)	Main Actions	Target Cells
IL-1α	17.5	pro-inflammatory	T-cell, B-cell, macrophage
IL-1β	17.5	pro-inflammatory	T-cell, B-cell, macrophage
IL-6	21-26	pro-inflammatory cell proliferation	T-cell, B-cell, activated NK-cell, hepatocyte, neuron
IL-8	8	pro-inflammatory chemotactic	Neutrophil, T-cell,
IL-10	17-21	anti-inflammatory	T-cell
IL-12	75(heterodimer of 40 and 35)	growth factor	NK-cell, B-cell, T-cell
RANTES	8	chemotactic pro-inflammatory	Monocyte, T-cell, eosinophil, mast cell
IFNα	19-29	anti-viral	most cells
TNFα	17	pro-inflammatory	most cells
LIF	32-45	haematopoietic	Monocyte, hepatocyte
PDGF	28(homodimer or heterodimer of A and B chains)	growth factor	fibroblast
MIF	12	chemotactic	macrophage
GM-CSF	18-32	haematopoietic	Monocyte, neutrophil, endothelial cell, fibroblast
M-CSF	45-90	haematopoietic	macrophage
G-CSF	19.6	haematopoietic	neutrophil
MIP-1α	8	chemotactic	Neutrophil, monocyte, basophil, lymphocyte subsets
TGF-α	5-20 (secreted forms)	growth factor	fibroblast
TGF-β	11.5-12.5	growth factor	fibroblast
bFGF	16	growth factor	fibroblast
IGF-1	6	growth factor	fibroblast

Interleukin-6 is a typical example of a multifunctional cytokine. First identified as a distinct hepatocyte stimulating factor involved in the induction of the acute phase response, it also induces differentiation of T-cells, B-cells, and macrophages, and is a potent growth factor for human myeloma cells (Akira 1992). Binding of IL-6 receptors induces the association of an accessory signal transducing molecule (gp130) which may also associate with several other cytokines including LIF (leukaemia inhibitory factor) and IL-11 (Kishimoto et al., 1995). This ligand-specific receptor with associated common signal transducer for multiple cytokines is found in most haematopoietic cytokine receptor systems and may explain the functional pleiotropy and redundancy of cytokines.

A principal chemoattractant for neutrophils (Peveri et al., 1988), IL-8 is also associated with various acute and chronic inflammatory states. In addition to bacterial-derived products such as LPS, both TNFα and IL-1 are capable of stimulating macrophage production of IL-8 (Strieter et al., 1990). Other actions of IL-8 include the induction of neutrophil polarisation and degranulation (Peveri et al., 1988) and altered adhesion receptor expression (down-regulating L-selectin and increasing CD11b/CD18 expression) (Detmers et al., 1991, Luscinskas et al., 1992). IL-8 binds to G-protein coupled receptors which have 7 transmembrane domains (Holmes et al., 1991).

Monocyte chemotactic factors include the TGFβ family of proteins, which also function in tissue remodelling during angiogenesis. There are 3 mammalian TGFβs which are often coexpressed in similar cell types and have similar actions *in vitro* (Derynck et al., 1985). TGFβ is released from platelets and macrophages combined

with a carrier protein (latent TGFβ) which is cleaved by low pH or certain proteases to become active TGFβ. *In vivo* TGFβ is immunosuppressive (Tsunawaki et al., 1988,Wahl et al., 1988) yet stimulates monocyte and fibroblast migration (Wahl et al., 1987, Wiseman 1988, Postlethwaite 1987) and induces production of extracellular matrix components by fibroblasts (Ignotz and Massague 1986, Fine and Goldstein 1987) and type II alveolar epithelial cells (Maniscalco et al., 1994, 1994a).

1.5 Extracellular Matrix

The extracellular matrix (ECM) is often considered as the scaffolding that supports the cellular elements of various tissues. It provides stability and support, separates epithelium or endothelium from interstitium, and thereby serves as a physical barrier by which tissues are compartmentalised. In addition, various cells e.g., fibroblasts, chondrocytes, endothelial cells and macrophages surround themselves in tissue culture by a pericellular ECM layer which excludes particles such as red blood cells and bacteria (Clarris and Fraser 1968) and may afford protection against compression *in vivo*.

Major ECM components include collagens, glycoproteins, and proteoglycans (Reichardt 1992). The structure and function of any connective tissue depends on the relative proportions of these constitutive molecules. Collagens are the main fibrous structures of connective tissues, characterised by 3 polypeptide chains, which form a triple helix basic unit (Prockop and Kivirriko 1995). For 80-90% of collagens in the body the triple helix represents the major part of the molecule (Ayad et al., 1994),

these are types I, II and III which are the classical fibril forming collagens. Types V and XI are also classified as fibrillar based on their homology to types I-III. The other collagens deviate considerably from this group both in structure and function within the ECM.

1.5.1 Glycoproteins

Matrix glycoproteins represent a heterogeneous group of structurally diverse molecules including fibronectin, tenascin, vitronectin and laminin, which comprise a core protein with carbohydrate covalently attached.

Fibronectin is a ubiquitous glycoprotein present in most extracellular matrices and in plasma. Like other glycoproteins, fibronectin is a mosaic protein composed of modular units (reviewed by Potts and Campbell 1994), and is secreted as a dimer which associates into disulphide-bonded oligomers and fibrils to produce the insoluble form in ECM, whereas the soluble form found in plasma remains mainly dimeric, polymerising when exudated from plasma. One of the main functions of fibronectin is the ability to promote cell adhesion, especially during wound healing and fibrosis (Grinell 1984). The site that promotes cell adhesion is located in the middle portion of the molecule, and involves an RGD sequence (Arg-Gly-Asp) which is a common motif recognised by integrins (reviewed by Gille and Swerlick 1996). Increased levels of fibronectin have been shown in lung injury and repair (Rennard et al., 1981, Broekelman et al., 1991), and soluble fibronectin has been shown to modulate cytokine production by monocytes (Beezhold and Personius 1992, Peat et al.,1995, Graves and Roman 1996).

Vitronectin, like fibronectin, also mediates various functions in coagulation, promotes adhesion to a variety of cell types (Felding-Haberman and Cheresh 1993) and is found in wound fluid (Grinnel et al., 1992). Elevated levels of vitronectin have been found in the synovial fluid of patients with rheumatoid arthritis (Rosenblum and Carsons 1996), and in the BAL fluid of patients with sarcoidosis (Eklund et al., 1992).

1.5.2 Proteoglycans

Proteoglycans are a diverse group of molecules comprised of a core protein attached to one or more covalently linked glycosaminoglycan (GAG) side-chains. Their diversity is determined by variation in the core protein, and in the type and size of the GAG chains. These GAG chains are composed of repeating disaccharide units, and include chondroitin sulphate, dermatan sulphate, keratan sulphate, and heparan sulphate. Heparan sulphate is the most abundant proteoglycan in basement membranes (Sivaram et al., 1995), and is thought to be important in inflammation, particularly through the sequestration of cytokines (Vlodavski et al., 1995, Jackson 1997).

1.5.3 Hyaluronan

Structurally related to heparan sulphate, hyaluronan is a high molecular weight linear non-sulphated GAG containing alternating N-acetyl-D-glucosamine and D-glucaronic acid residues linked by β(1-4) and β(1-3) bonds. In solution it exists in a

random coiled configuration, which is highly hydrated and thus has an important role in tissue hydration.

Hyaluronan is a widely distributed ECM component, it acts in embryonic development (Fisher and Salrush 1977), tumour growth (Sy et al., 1996, Naor 1997), wound healing (Bentley 1967,1968), as a provisional matrix for the support of cell migration and growth (Weigel et al., 1986, Peck and Isacke 1996), and in combination with other proteoglycans is a major component of pericellular matrices (Hedman et al., 1982, Lee et al., 1993).

During inflammation hyaluronan undergoes dynamic regulation, elevated levels of hyaluronan have been found in the serum of patients with rheumatoid arthritis (Emlen et al., 1996), and sepsis (Laurent et al., 1996a). Whereas reduced levels and altered molecular weight forms of hyaluronan have been found in the synovial fluid of patients with rheumatoid arthritis (Dahl et al., 1985). During inflammation, hyaluronan and fibronectin accumulate in the fibrin provisional matrix and have been recovered from the air-lung interface after the onset of acute lung injury (Bray et al., 1991).

1.6 Receptors for Hyaluronan

Receptors for hyaluronan include RHAMM (receptor for hyaluronan mediated motility) and CD44. RHAMM is expressed at low levels on many quiescent cells, including macrophages (Turley 1992), and as the name suggests, is involved in cell motility (Hall et al., 1994).

The principal receptor for hyaluronan is thought to be CD44, the most common form is the smallest (80-95kDa), and is a single chain molecule composed of a distal extracellular domain, which contains the ligand binding sites, a transmembrane-spanning domain, and a cytoplasmic tail. As both hyaluronan and CD44 are ubiquitously expressed it is probable that extensive variations in the structure of CD44 govern the adhesive interactions between them.

CD44 is subject to post-translational modifications, such as glycosylation (Goldstein et al 1989), and phosphorylation (Neame and Isacke 1992). In addition, 10 alternative splice regions within the genomic organisation (exons v1-v10) provide CD44 with great molecular diversity. Within the variable region, there are a further 4 potential N-glycosylation sites, and several potential O-glycosylation sites, in addition to a potential insertion motif (serine-glycine) for glycosaminoglycans (Screaton et al., 1993).

The cytoplasmic tail is highly conserved (Goldstein et al., 1989, Stamenkovic et al., 1989) and contains 6 potential phosphorylation sites, of which some are constitutively phosphorylated (Carter and Wayner 1988, Camp et al 1991). In addition, there are consensus phosphorylation sites for protein kinase C and protein kinase A (Camp et al 1991). Further diversity is provided by the 4 serine-glycine motifs in the extracellular domain, which can be modified by heparan sulphate (Tanaka et al., 1993) and chondroitin sulphate (Jalkanen et al., 1988, Stamenkovik et al., 1989). These modifications could possibly result in altered ligand specificity (Jalkanen and Jalkanen 1992). In fact, CD44 can act as a receptor for several other ECM components including collagen (Carter and Wayner 1988), fibronectin, and

laminin (Jalkanen and Jalkanen 1992). In addition some non-ECM ligands have been reported including osteopontin (Weber et al., 1996).

1.7 ECM, and cell interactions

In addition to its structural role the ECM provides a context in which cellular reactions can occur. Matrix proteins also confer signals regulating cell growth and activation. Leukocyte adhesion to ECM, as previously mentioned, is one of the first influences on monocytes as they extravasate. It is important as a cell-activating event, and adhesion to different ECM proteins has been reported to affect;

- Spreading (Edwards et al., 1993).
- Cytoskeletal changes (Mueller et al., 1989)
- Focal adhesion assembly (reviewed by Otey and Burridge 1990).
- Stimulation of proliferation and migration (Adams and Shaw 1994, Vlodavski et al., 1995).
- Induction of cytokines and chemokines (Eierman et al., 1989, Neese et al., 1994, Formica et al., 1994, Peat et al., 1995).
- Alterations in phagocytic behaviour (Newman and Tucci 1990, Suzuki and Yamaguchi 1993).
- Alterations in reactive oxygen and nitrogen intermediates (Formica et al., 1994, Gudewicz et al., 1994).

1.8 ECM, Cytokines and Cell Responses

Several cytokines including IL-3, GM-CSF and several forms of fibroblast growth factor (FGF) are functionally active when bound to proteoglycans in ECM (Nathan 1991). IL-8 becomes a more potent chemoattractant when bound to heparan sulphate

(Webb et al., 1993). The structure of proteoglycans is ideal for the presentation of cytokines because of the long highly charged and hydrophilic GAG chains. Like the selectins these chains can reach the periphery of the glycocalyx where they may capture cytokines released from the endothelium or underlying tissue and present them to loosely tethered leukocytes (Tanaka et al., 1993). The structural variation of proteoglycans on endothelium may add specificity to these interactions by regulating which cytokines are retained. So site-specific or activation-specific endothelial proteoglycans may bind and present different proadhesive cytokines to particular subsets of leukocytes (Adams and Shaw 1994).

In addition, many cell responses to cytokines are altered when the cell is bound to ECM proteins through initiation of adhesion-dependent signal transduction e.g., TNFα acts as a secretagogue for neutrophils only when adhered to ECM via β2 integrins (Nathan and Sanchez 1990, Shappel et al., 1990, Richter et al., 1990).

1.9 ECM Turnover

Events in wound healing can be divided into 3 stages; inflammation, early and late granulation tissue formation (Gailit and Clarke 1994). Each stage requires the carefully controlled synthesis and removal of the various structural proteins, glycoproteins and proteoglycans of the ECM. The degradation of ECM is achieved through the interactions between cells, cytokines, proteases, and protease inhibitors. During inflammation neutrophils and macrophages release proteases and reactive oxygen intermediates (Laskin and Pendino 1995). These products may be involved in the degradation of ECM components exposed after endothelial injury (Baker et al.,

1989). Macrophage production of the various proteases capable of degrading ECM components, including matrix metalloproteases, appears to be regulated by contact with the ECM itself (Shapiro et al., 1993) and cytokines (Wahl and Corcoran 1993).

Although leukocytes need to be able to degrade ECM in order to respond to inflammatory stimuli and migrate successfully through ECM barriers, the uncontrolled production of these degradative products can result in excessive ECM disruption. As ECM can bind and sequestrate growth factors necessary for repair of injured tissue these may be released during this degradation, either hindering the resolution of inflammation or leading to disease pathogenesis. Clearly there is a complex interplay of mediators in inflammatory processes, involving inflammatory cells, cytokines and ECM components. In the lung, the abnormal regulation of these processes may result in parenchymal lung destruction.

1.10 Inflammation in the lung

Historically, the lung was perceived as an organ primarily involved in gas exchange, however it also plays an important role in mediating host defence. Upon exposure to inhaled immunogens one response of pulmonary epithelial cells in addition to their role in mucociliary clearance through the secretion of mucous and efficient ciliary movement in the upper airways, is synthesis and release of a number of cytokines and other inflammatory mediators. Alveolar macrophages and leukocytes within the extensive alveolar capillary network also provide an important defence against blood borne immunogens. Following generation of an inflammatory response and the

clearance of the initiating agent, inflammation is resolved and normal repair and tissue remodelling re-establishes normal lung function without the development of a pulmonary fibrotic response. In chronic inflammation within the lung, the process of healing through scar formation or fibrosis may reduce the capacity for gas exchange and have serious consequences for normal lung function that might ultimately affect host survival.

1.11 Adult Respiratory Distress Syndrome

The most severe form of acute lung injury is the adult respiratory distress syndrome (ARDS), which develops most often 24-72 hours after the provoking insult (Donnelly and Haslett 1992). Originally described in 1967 (Ashbaugh), ARDS is an example of an acute inflammatory response that frequently progresses to a fibrotic condition. Initially, it is characterised by a leakage of proteinaceous fluid into the interstitium and airspaces. Type I epithelial cells which facilitate the exchange of gas across their thin cytoplasm in normal lung function, provide a barrier to the movement of fluid into the airspaces (Bachofen et al., 1982). In ARDS, extensive injury of these type I epithelial cells results in the disruption of the epithelial layer and allows the leakage of fluid and proteins into the alveolar spaces, this is accompanied by an excessive neutrophil influx. During disease progression, type II epithelial cells, which are more resistant to injury, proliferate and replace type I cells (Riede et al., 1979). In response to common precipitating insults, including multiple trauma, pancreatitis, and endotoxaemia, a microenvironment that favours cell injury is created in part through the release of pro-inflammatory mediators such as TNFα

(Armstrong and Millar 1997) and IL-1 (Pugin et al., 1996), and chemotactic agents such as IL-8. These cytokines prime and activate neutrophils and other inflammatory cells to release histotoxic agents including proteases and reactive oxygen species, which are capable of damaging extracellular matrix components exposed after the destruction of epithelial or endothelial cells (figure 1.1). Analysis of bronchoalveolar lavage fluid obtained from patients with ARDS has revealed elevated levels of TNFα (Millar et al., 1989) and IL-1β (Suter et al., 1992), both of which can stimulate production of IL-8 by endothelial cells (Huber et al., 1991), and macrophages (Strieter et al., 1990). In fact, in addition to elevated IL-8 levels in bronchoalveolar lavage fluid from patients with ARDS (Miller et al., 1992), elevated levels of IL-8 have been found in the lavage fluid of patients at risk of ARDS who subsequently progressed to ARDS (Donnelly et al., 1993).

Some patients with ARDS recover rapidly as alveolar structure and function are regained, however ARDS has an associated mortality rate of 50%. In those patients who die from ARDS, a fibroproliferative process occurs, which is poorly understood, but is characterised by fibrin deposition in the alveolus. In addition, fibroblasts and myofibroblasts proliferate within the alveolar wall, and migrate through the denuded basement membrane depositing scar tissue matrix proteins (Raghu et al., 1985) which leads to fibrosis (Bitterman et al., 1992). The subsequent proliferation of mesenchymal cells within the alveoli and the formation of new capillaries, results in the obliteration of normal lung architecture (Tomashefski et al., 1983).

It is probable that the neutrophil influx during ARDS and consequent lung damage results from mediators produced by cells already resident in the lung. Under normal

conditions and during chronic inflammation, the most abundant immune cell in the lung is the macrophage. However, little is known about the mechanisms of macrophage activation in this type of inflammatory environment.

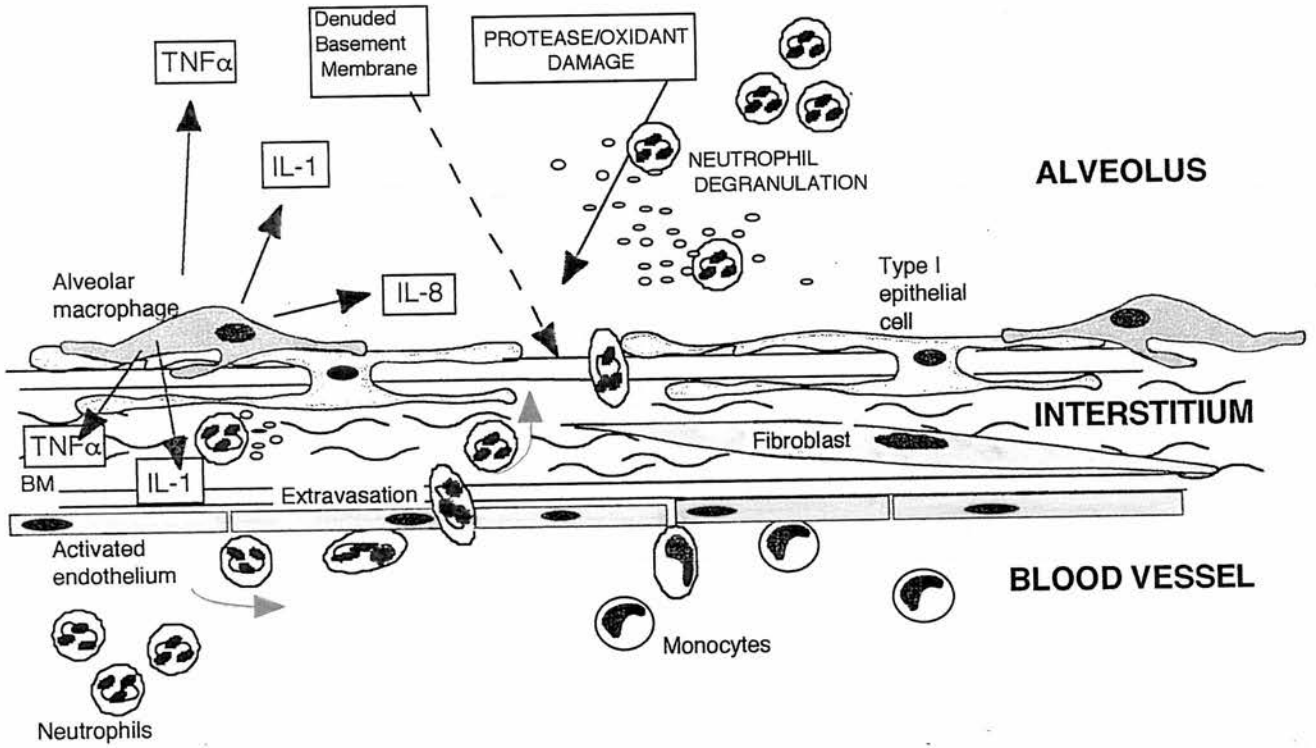


Figure 1.1

During acute inflammation, such as that seen in ARDS, a micro-environment that favours tissue injury is created. Alveolar macrophages produce chemokines and cytokines, resulting in the activation of endothelial cells, and the recruitment of leukocytes. Type I epithelial cells, are destroyed resulting in exposure of the basement membrane. In addition to macrophage proteases and oxidants, neutrophil degranulation products contribute to epithelial and endothelial damage, resulting in leakage of fluid and proteins into the lung. These oxidants and proteases may also alter ECM components within the interstitium and basement membrane, directly affecting cells that come into contact with these ECM components.

1.12 Aims

The work presented in this thesis aimed to characterise the monocyte/macrophage activation seen in inflammatory environments such as that seen in ARDS.

Initially I undertook a comparative phenotypical analysis of monocytes and monocyte-derived macrophages, with the aim of investigating alveolar macrophages obtained from patients with ARDS and those at risk of developing ARDS. These studies are described in chapter 3.

Secondly, I developed an *in vitro* model for assessing the effects of altered composition on inflammatory macrophage phenotype using human peripheral blood monocytes and monocyte-derived macrophages. Hyaluronan, a key ECM component was found to have pro-inflammatory actions on monocytes and was investigated further (described in chapter 4).

Thirdly, the clinical relevance of pro-inflammatory actions of hyaluronan was investigated, by quantifying hyaluronan in clinical material (bronchoalveolar lavage fluids and lung biopsy specimens), obtained both from patients with ARDS, and patients at risk of developing ARDS (described in chapter 5). Finally, the pro-inflammatory effects of hyaluronan on monocytes and macrophages were further characterised through receptor blocking studies and studies on cell survival.

The overall intent was that these combined approaches would provide some insight to the role of the extracellular environment in determining inflammatory monocyte/macrophage function and the implications for inflammatory disease progression.

Chapter 2

2.1 Reagents.

Tissue culture media, including Dulbecco's Modified Eagles Medium (DMEM), Iscove's Modified Dulbecco's Eagles Medium, Hanks balanced salts solution, L-glutamine, penicillin and streptomycin, were obtained from Life Technologies (Paisley, UK). Sterile disposable tissue culture ware was from Falcon Plastics, Becton Dickinson, (Oxford, UK). Dextran T500 and Percoll were obtained from Pharmacia Biotechnology (St. Albans, Herts.), all other chemicals, unless otherwise stated, were obtained from Sigma Diagnostics (Poole, UK).

2.2 Materials and Methods

2.2.1 Alveolar macrophage isolation and culture

Bronchoalveolar lavage (BAL) was performed on normal volunteers (smokers and non-smokers), patients at risk of ARDS, and patients with ARDS. BAL was performed by Drs. S.C. Donnelly, P. Reid, and D. Morrison. Briefly, 3 x 60ml 0.9% NaCl was instilled into the right middle lobe and gently aspirated immediately. BAL fluid was then strained through sterile gauze to remove mucous, and then spun at 400 x g at 4°C for 10 minutes to recover cells. Cell numbers and viability were determined by trypan blue exclusion using a haemocytometer (section 2.2.17). Cells were washed in HBSS, and either re-suspended in PBS with 0.2% BSA, and 0.1% sodium azide for flow cytometry (section 2.2.17), or re-suspended in Iscove's

modified Dulbecco's Eagles medium (Iscove's DMEM) and allowed to adhere for 1hr (37°C, 5%CO_2) to tissue culture plastic. Adherent cells were washed after 1hr with gentle pipetting with HBSS to remove non-adherent cells and cultured in Iscove's DMEM with 10% myoclone + foetal calf serum.

2.2.2 Flow Cytometry

Cells for flow cytometric analysis were resuspended at 2 x 10^6/ml in cold PBS with 0.2%BSA, and 0.1% sodium azide, and 50µl cell suspension was used per test. To block non-specific binding of antibody through Fc receptors, cells were pre-incubated with 20% normal rabbit serum for 20 minutes. All antibodies were used at saturating concentrations, as determined by titrations.

2.2.3 Indirect technique

Antibodies (50µl per test, listed in table 2.2) were added to the cell pellet in 96 well flexible assay plates (Falcon), and incubated on ice for 30 minutes. Cells were then washed twice in PBS/BSA/Azide to remove unbound antibody, centrifuged at 225 x g and resuspended in fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin (Dako corporation, High Wycombe, Bucks.) diluted 1:40 in PBS/BSA/Azide. Alternative second layer conjugates were used on autofluorescent macrophages; Tri-colour (Bradsure Biologicals, now TCS Biologicals, Botolphclaydon, Bucks.), and Quantum Red (Sigma). Following a 30 minute incubation on ice with the second layer, cells were washed twice (as before), and

either resuspended in PBS/BSA/Azide and analysed immediately, or fixed in 3% formaldehyde in PBS and kept at 4°C, protected from light until analysed.

2.2.4 Direct technique

Phycoerythrin-conjugated anti-CD14 monoclonal antibody was added to the cell pellet and incubated for 30 minutes on ice. Following two washes (as before) cells were analysed immediately. Flow cytometric analysis was performed using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, Beds.).

2.2.5 Autofluorescence Quenching

Crystal violet (Sigma) was dissolved (2mg/ml) in sterile PBS and filtered. Cells were treated with crystal violet prior to indirect immunofluorescent staining (section 2.2.3) then fixed in 3% formaldehyde/PBS. Alternatively, cells were subject to indirect staining, fixed in 4% paraformaldehyde, permeabilised with n-octyl-β-D-glucopyranoside (OG, Sigma), then treated with crystal violet according to Hallden et al., (1991). Flow cytometric analysis was performed using an EPICS Profile II flow cytometer, as before.

Table 2.1 Antibodies for Flow Cytometry

Antibody	Flow dilution	Supplier
MOPC	Neat, supernatant	MOPC31C, hybridoma, ECCC
CD3	Neat	UCHT1, hybridoma, ECCC
CD11a	1:8	Serotech, Oxford
CD11b	1:4	Serotec, Oxford
CD11c	1:4	SAPU, Carluke, Lanarkshire
CD14	1:4, supernatant	UCHM1, hybridoma, P.Beverley.
CD14-PE	1: 25,	Tuk-4, Dako
CD15	1:8	Serotec, Oxford
CD16	Neat, supernatant	3G8 hybridoma, J.Unkeless,
CD43	1:4	Serotec, Oxford
CD44 (5A4)	Neat, supernatant	5A4 hybridoma G.Dougherty
CD44 (7F4)	Neat, supernatant	7F4 hybridoma, G.Dougherty
CD44 (v3)	1:500	Becton Dickinson
CD44 (v7-8)	1:100	Bender
CD44 (v10)	1:1000, Ascites	G.Dougherty, Los Angeles
CD51	1:4	Becton Dickinson
CD62L (LEU 8)	1:8	Becton Dickinson
CD66b (HLA-DR)	Neat, supernatant	SAPU, Carluke, Lanarkshire
CD67	1:200	Becton Dickinson
CD69 (LEU23)	1:4	Becton Dickinson
CD71	1:8	Becton Dickinson

2.2.6 Monocyte isolation

Mononuclear cells were isolated from peripheral blood from normal donors. Blood (40ml) was obtained by venipuncture and anti-coagulated with 0.38% sodium citrate in sterile 50ml tubes (Falcon). Tubes were then centrifuged at 300 x g for 20 minutes (MSE Mistral 3000 Sanyo Gallenkamp, Loughborough UK) at room temperature (22°C) to yield a cell-rich pellet and a platelet-rich upper layer.

Autologous serum was prepared by adding 330µl calcium chloride (0.32%) to 15ml platelet-rich plasma (PRP) in glass tubes, and incubating at 37°C. Platelet poor plasma (PPP) was prepared by centrifuging the remaining PRP at 2500 x g for 20 minutes.

Erythrocyte sedimentation was performed by addition of 2.5ml of warm (37°C) 6% Dextran T500 per 10ml of leukocyte-rich pellet. Cell pellets were made up to 50ml with warm saline and allowed to sediment at room temperature for 30 minutes.

Isotonic Percoll was prepared by adding 3ml of 10x PBS without calcium or magnesium to 27ml Percoll (Pharmacia), and discontinuous density gradients were prepared by overlaying an 81% isotonic Percoll layer with a 70% layer. The leukocyte rich upper layer was washed in saline and re-suspended in 55% isotonic Percoll which was then layered onto the gradients. Gradients were centrifuged at 720 x g for 20 min (Dooley et al., 1981).

Mononuclear leukocytes were aspirated from the 55%/70% interface and washed twice in HBSS without calcium or magnesium.

Cell number and viability were assessed by trypan blue exclusion on a haemocytometer (section 2.2.17). Purity was assessed by flow cytometry, according to forward scatter and side scatter characteristics (section 2.2.4)(figure 2.1a).

2.2.6a) Adherence isolation of monocytes

Monocytes were isolated by adherence to autologous plasma-coated tissue culture plates, adapted from Ackerman (1978). 10cm petri dishes were coated using 10% autologous PPP in PBS without calcium or magnesium and incubated for 30 minutes at 37°C. The plates were washed in PBS to remove any remaining platelets before adhering the cells. Mononuclear cells were allowed to adhere for 1 hour (37°C, 5%CO_2) in Iscove's DMEM with 10% autologous serum, after which time non-adherent cells were removed by washing four times in warm HBSS. Adherent cells were detached using 5mM EDTA in PBS with 2% autologous serum for 5 min at 37°C. Cells were then washed in Iscove's DMEM with 10% serum and additives (penicillin, 0.5u/ml, streptomycin, 0.5U/ml) (monofeed), and checked for purity and viability (section 2.2.17) (figure 2.1b).

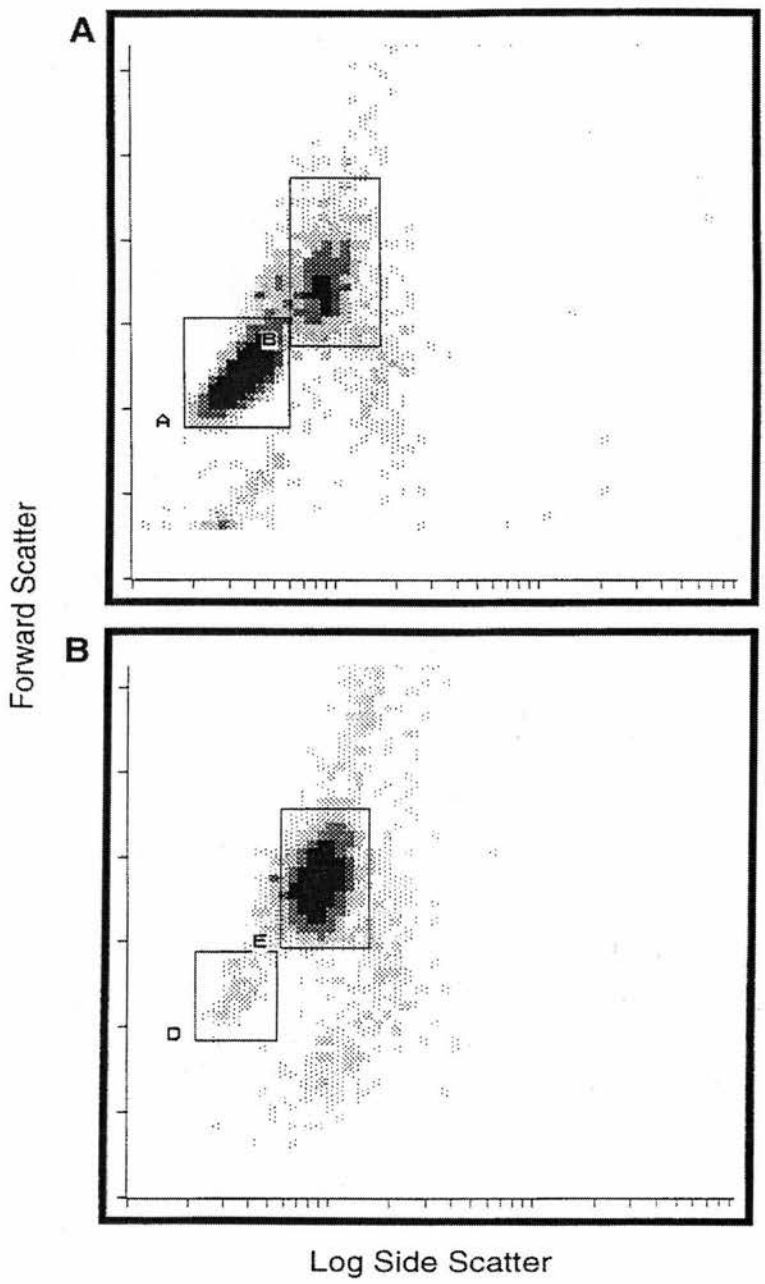


Figure 2.1

A. Mononuclear leukocytes from the 55%/70% interface of discontinuous Percoll density gradients were checked for purity by flow cytometry on the basis of forward and side scatter characteristics; monocytes are shown in gate B, whereas gate A shows lymphocytes.

B. Monocytes isolated by adherence to plasma-coated plates were also assessed for purity by flow cytometry, according to forward scatter and side scatter characteristics.

2.2.6b) Counter Current Centrifugal Elutriation

Cell elutriation was performed using a J-21M/E Beckman centrifuge (Beckman Instruments (UK.) Ltd, High Wycombe, UK.) with a JE-6 elutriator rotor with flow rate regulated by a peristaltic pump (figure 2.2). The leukocyte-rich layer, prepared by Dextran sedimentation was loaded into the elutriation chamber at a rotor speed of 2200rpm with a fixed flow rate of 6ml/min. The pump speed was gradually increased, allowing the cell interface in the elutriation chamber to settle between each increase, and cells were elutriated according to their size and density. Cell populations were monitored through regular sampling and flow cytometry (section 2.2.4). Cells were collected in 50ml fractions, washed in HBSS and checked for viability (section 2.2.17).

Initial elutriation runs performed at room temperature in the presence of autologous plasma resulted in low cell yields after 24 hours in culture, or total cell death. Although the elutriator tubing was sterilised with ethanol prior to each run, accumulation of plasma proteins from different donors could result in cell activation and possibly cell necrosis. Autologous plasma was substituted with foetal calf serum (FCS) and the buffer and rotor cooled to 4°C prior to use.

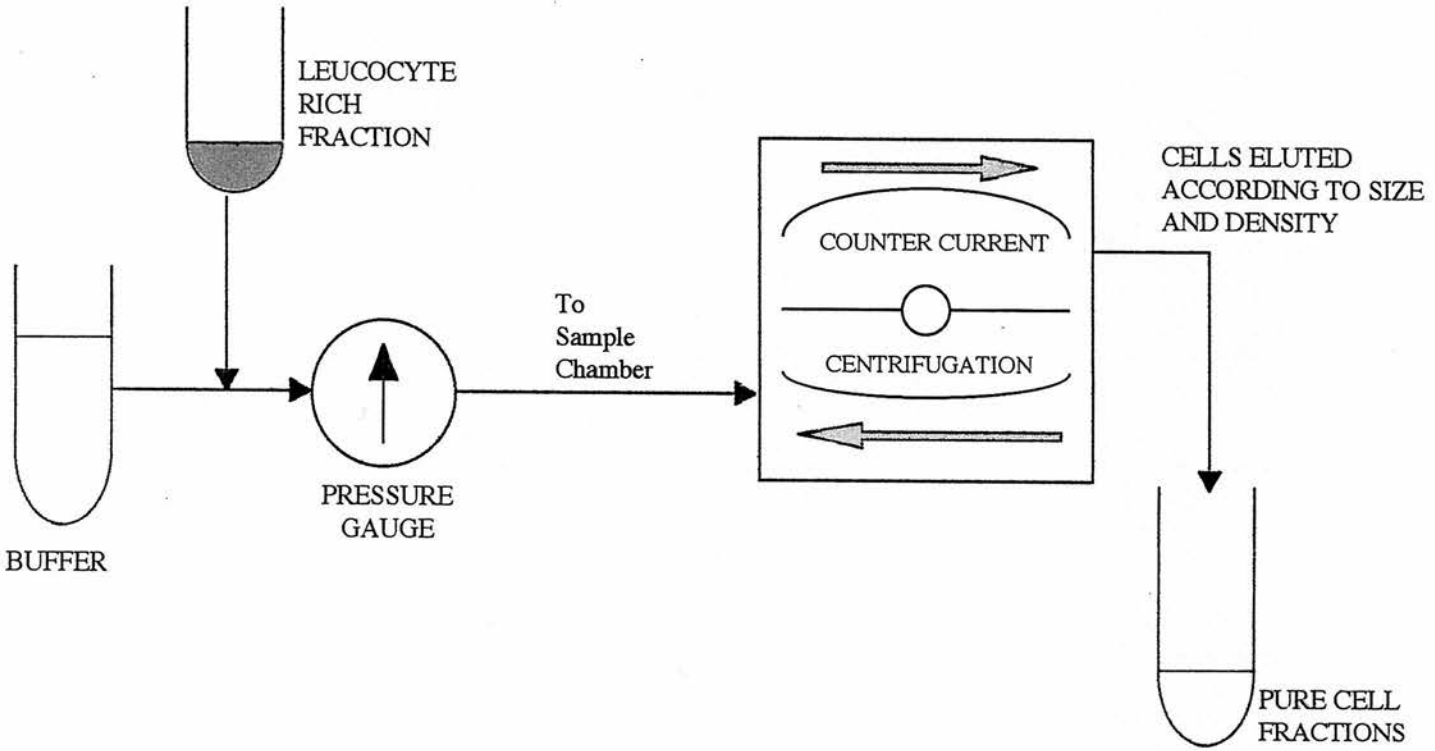


Figure 2.2 Schematic representation of cell isolation using counter current centrifugal elutriation.

These alterations resulted in a reduction of total elutriation times and increased viable cell yields.

2.2.6c) Comparison of Isolation Techniques

To determine if one leukocyte isolation method resulted in better yields and survival rates than the other, a comparison was made. Flow cytometric analysis (figure 2.3) showed that when compared to Percoll isolated neutrophils, elutriated neutrophils had reduced expression of CD62L, suggesting that these cells were more activated. In a separate experiment (data not shown) rates of neutrophil apoptosis were found to be increased for the elutriated neutrophils. These data together would suggest that elutriation was resulting in neutrophil activation and decreased cell survival rates, when compared with Percoll isolated cells. Monocyte survival over 6 days in Teflon was comparable for the two different isolation procedures (table 2.1)

Table 2.1 Comparison of Elutriated and Percoll Separated cell survival

Donor	Method	Day 1 Yield ($x10^6$)	Day 6 Yield ($x10^6$)	% Survival
1	Percoll	20	0.01	0.05
	Elutriated	20	0.32	1.6
2	Percoll	6.0	0.6	10.0
	Elutriated	7.0	0.33	4.7
3	Percoll	20	3.3	16.5
	Elutriated	10	2.0	16.0

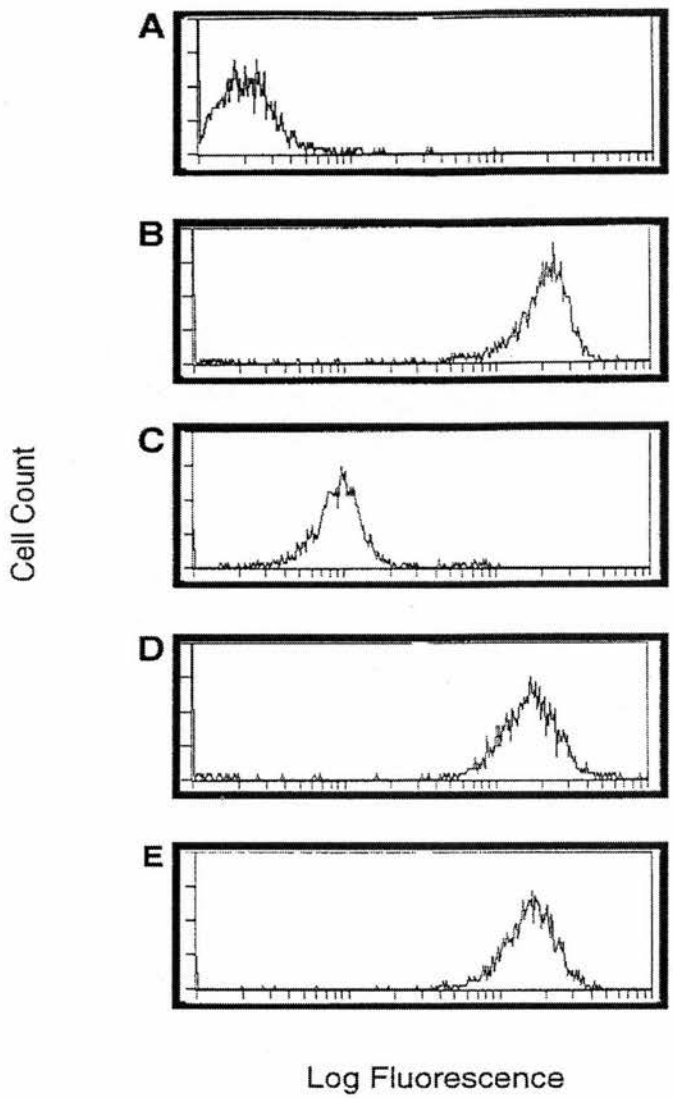


Figure 2.3

Neutrophils isolated by elutriation were compared with neutrophils isolated by Percoll density gradients for expression of CD62L, and CD11b, through flow cytometric analysis, to assess the cell activation between techniques. A; negative control, B; Percoll isolated neutrophil CD62L expression, C; elutriated neutrophil CD62L expression, D; Percoll isolated neutrophil CD11b expression, E; elutriated neutrophil CD11b expression.

However, elutriated monocytes cultured adherent to tissue culture plastic (data not shown) did not adhere firmly and by day 6 were easily washed off. Considering these data, and due to the nature of elutriation, which has multiple opportunities for system errors and contamination, Percoll separation was chosen as the preferable method for reliable yields of monocytes.

2.2.7 Monocyte culture

Monocytes were cultured for 6 days in monofeed at a density of 10^6 cells/ml, either adherent to tissue culture treated plastic (Falcon) or in suspension in hydrophobic Teflon foils (section 2.2.8.a). Medium was changed at day 3 for adherent cultures.

2.2.8 Preparation for Cell Culture

2.2.8a) Teflon Foils

Hydrophobic Teflon foils were prepared by taking 5cm squares of Teflon (Chemfab, Handforth, Cheshire, UK) and folding 1cm in of each edge (figure 2.4). Corners were then sealed using a modified HM3000 Impulse heat sealer (Hume Martin Ltd, London, UK), and the Teflon foils were autoclaved prior to use.

2.2.8b) ECM Plates

For ECM plates, tissue culture treated plastic was coated overnight at 4°C with 1mg/ml hyaluronan, 10μg/ml heparan sulphate, 10μg/ml fibronectin, or 10μg/ml vitronectin, in PBS.

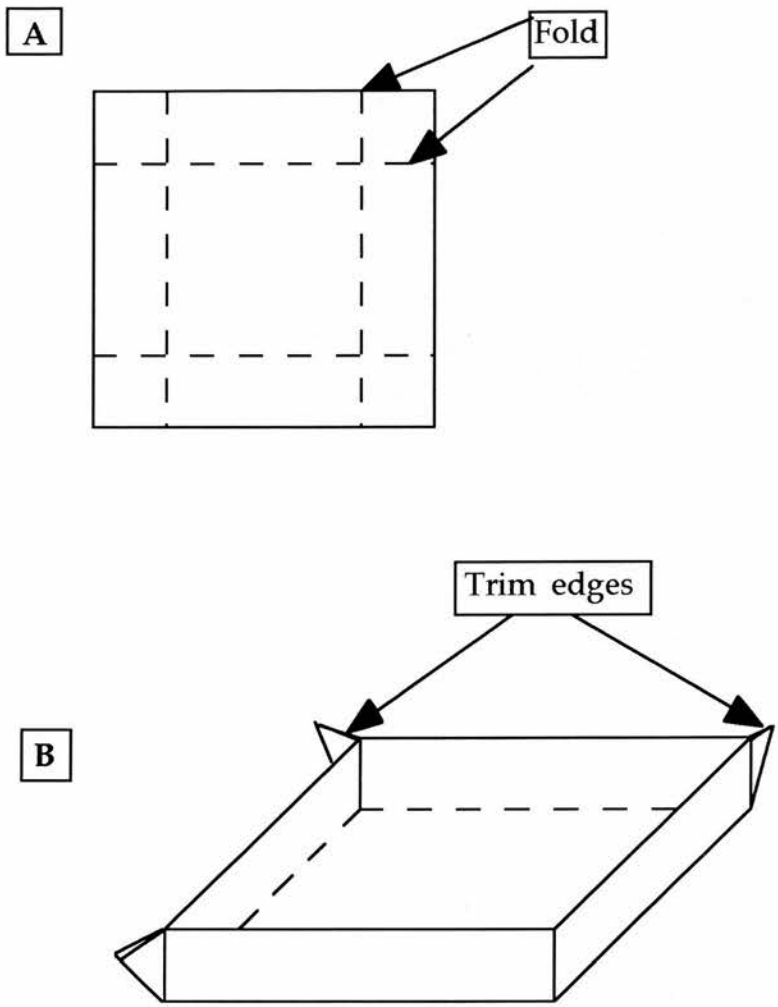


Figure 2.4

Hydrophobic Teflon trays were prepared with 5cm squares of Teflon, and 1cm was folded in at each edge (A). Corners were then sealed using a modified HM3000 Impulse heat sealer (B). Edges were trimmed and the teflon trays were autoclaved prior to use.

2.2.9 Morphological Assessment of Apoptosis

Apoptosis was assessed using Acridine Orange (Sigma) which intercalates with DNA to emit a green fluorescence. During apoptosis chromatin condenses and apoptotic monocytes are easily recognised by the bright green stained nucleus. Acridine Orange (0.5μl) was added directly to the wells to give a final concentration of 1μg/ml, this was sufficient to stain the cells without high background fluorescence.

2.2.10 Immunohistochemical Staining Lung Tissue Sections

Paraffin embedded lung biopsy samples, kindly provided by Edinburgh University Pathology department, were cut into sections and mounted on Poly-L-Lysine coated slides. These were de-waxed in xylene, sequentially re-hydrated in 100% ethanol, 70% ethanol, 55% ethanol, then washed in running water. Slides were blocked for endogenous peroxide with 1% H_2O_2 (15 minutes) then blocked (20 minutes) for non-specific binding in Tris buffered saline (pH 7.6) 1% bovine serum albumin (BSA). Slides were then incubated (overnight, 4°C) with biotinylated hyaluronan binding region (HaBR, kindly provided by Mike Bayliss, Kennedy Institute, London) diluted in TBS 1% BSA. Slides were washed in TBS then incubated (1 hour) with streptavidin-biotin-HRP (Dako corporation, High Wycombe, Bucks.) diluted 1:500 in TBS containing 1% BSA. After further washing, staining was visualised using 3,3' Diaminobenzidine (DAB, 500μg/ml in Tris/HCl pH 7.6 with 1% H_2O_2), this substrate reaction was stopped by rinsing slides with water. Slides were then

counter-stained with Haematoxylin blue prior to re-hydrating sequentially in 55%, 70%, and 100% ethanol, finally slides were rinsed in xylene, and mounted.

To control for non-specific staining, HaBR was adsorbed (overnight, at 4°C) onto high binding flat bottom 96 well plates (Corning Costar, high Wycomb, Bucks.) pre-coated with hyaluronan (1mg/ml). Slides used for negative controls were treated in the same manner as before using this HaBR that had been pre-adsorbed onto hyaluronan in solid phase.

2.2.11 Cell adhesion assay

A quantitive method for assessing cell adhesion was modified from Oliver et al. (1989). High binding 96 well plates (Maxisorp, Nunc Life Technologies, Paisley) were pre-coated with hyaluronan (1mg/ml), fibronectin (10μg/ml), keyhole limpet haemocyanin (10μg/ml)(Sigma), or sterile BSA (0.2%). Monocytes (at 2 x 10^6/ml, or 0.5 x 10^6/ml) were allowed to adhere for 30 minutes at 37°C in HBSS, or HBSS without calcium or magnesium in the presence of 5mM EDTA. Plates were then washed twice in HBSS and fixed with 4% formalin in PBS. Remaining cells were then stained with 1% methylene blue for 30 minutes, washed with distilled water, and lysed in 0.1N HCl. Optical densities were determined at 630nm (Dynatech MR5000 plate reader Billinghurst, Kent). Alternatively, assays were assessed by microscopy, the number of adherent cells in 4 randomly selected fields were counted in each well. The edges of the wells were avoided, where cells remained adherent to different substrates throughout the washing procedures.

2.2.12 Cell Bound ELISA

Mononuclear cells (5 x 10^6/ml) were plated onto a 96 well tissue culture plate (150µl/well) and allowed to adhere (37°C, 5% CO_2) for 1 hour. After washing in HBSS to remove non-adherent cells, monocytes were cultured overnight in monofeed. Adherent cells were then washed twice with PBS 0.1%BSA and incubated with antibodies for 1 hour at room temperature. After washing three times with PBS/BSA/Azide, cells were either fixed with 4% paraformaldehyde or left unfixed then incubated with β-galactosidase-conjugated goat anti-mouse IgG (1:1000, Sigma) for 1 hour at either 37°C or room temperature. The enzyme β-galactosidase was chosen as an alternative to peroxidase-conjugated enzyme systems, to avoid any interference from endogenous peroxidase activity. After washing again, o-nitrophenyl β-D-galactopyranoside (ONPG) substrate (4mg/ml) in 0.1M sodium phosphate with 0.1M $MgCl_2$/4.5M β-mercapto-ethanol was added and colour allowed to develop. Substrate reaction was stopped using 1M $NaCO_3$, and after transferring the supernatant to a fresh 96 well ELISA plate, absorbance was read at 405nm using a plate reader (MR5000 Dynatech, Billinghurst, Kent.).

2.2.13 ELISA

2.2.13a) Antibodies

Paired TNFα monoclonal antibodies were from Boehringer Mannheim.
IL-8 rabbit anti-human polyclonal capture antibody was from AMS (Oxon, UK), alternatively, a murine hybridoma-derived IL-8 monoclonal capture antibody was obtained from R&D Systems (Oxon, UK), goat anti-human polyclonal detection IL-8

antibody was from R&D Systems (Oxon, UK). Concentrations were determined by cross-titrations.

IL1β and TGFβ ELISA kits obtained from R&D Systems.

2.2.13b) Standards

Recombinant human IL-8 (endothelial cell derived) and recombinant human TNFα were obtained from R&D Systems.

2.2.13c) Solutions for ELISA

Coating buffer:	$Na_2CO_3/NaHCO_3$, pH 9.6, carbonate bicarbonate capsules (Sigma)
Blocking buffer:	1% Bovine serum albumin (Fraction V, Sigma) in coating buffer
Wash buffer:	Tris-HCl (20mM)/NaCl (150mM), pH 7.4 Tween 20 (0.1%)
Sample buffer:	Tris-HCl (20mM), pH 7.4, NaCl (150mM) 1%BSA
Substrate buffer:	Sodium acetate-citrate 100mM pH 4.9
Substrate stock:	TMB (3,3',5,5'-Tetramethylbenzidine) dissolved in DMSO (dimethylsulfoxide) 10mg/ml
Substrate solution:	100µl TMB stock solution in 10ml substrate buffer with 15µl H_2O_2(30%)
Stop solution:	1M H_2SO_4

2.2.14 ELISA Protocols

2.2.14a) IL-8 ELISA

High binding flat bottom 96 well plates (Corning Costar, High Wycombe, Bucks.) were coated overnight at 4°C with the capture IL-8 antibody diluted in coating buffer, then blocked in coating buffer with 1%BSA. After each step, unless otherwise stated, washing with TBS-Tween 20 (4 x 200µl/well) was performed. Standards (20ng-0.02ng) and samples were then diluted in TBS 1%BSA and incubated at 4°C overnight (16 hours). The detection antibody was added and incubated for 2 hours at room temperature. Rabbit anti-goat IgG-peroxidase conjugate (100µl/well, Sigma) was then incubated for 2 hours at room temperature, this was followed by the addition of substrate solution (TMB in sodium acetate citrate with H_2O_2, 100µl/well). The colour was allowed to develop protected from light, and stopped with 100µl H_2SO_4/well. The optical density was measured with a plate reader (Dynatech MR5000, Billinghusrt, Kent) at 450nm with a correction filter at 630nm.

2.2.14b) TNFα ELISA

High binding flat bottom 96 well plates (Corning Costar, High Wycombe, Bucks.) were coated with capture anti-TNFα in coating buffer, at 35°C for 2hr then blocked in blocking buffer. Samples were diluted 1:5 in sample buffer and were incubated with standards overnight at 4°C. The detection antibody was incubated for 4hr at room temperature. Substrate reaction and optical density were performed as described in section 2.2.14a.

2.2.14c) TGFβ ELISA

ELISA kit manufacturers protocol was followed. Prior to assaying, samples for TGFβ ELISA were acid-activated to activate latent TGFβ to the immunoreactive form. To 100µl cell culture supernatant 20µl 1N HCl was added, vortexed and incubated for 10 minutes. This was then neutralised by adding 20µl 1.2N NaOH/0.5M HEPES and vortexing. All incubation steps were performed at room temperature. Samples were then incubated in the pre-coated anti-TGFβ immunoassay plates provided, for 3 hours. After washing, HRP-conjugated anti-TGFβ was added to each well. After 1.5 hours, the plates were washed, then incubated with substrate solution. The substrate reaction was stopped with 1M H_2SO_4, and read as described in section 2.2.14a.

2.2.14d) IL-1β ELISA

The manufacturer's protocol was followed, all incubation steps were performed at room temperature. Briefly, samples were diluted (1:5) and incubated in the pre-coated anti IL-1 immunoassay plates provided, for 2 hours. After washing, HRP-conjugated anti-IL-1 was added to each well. After 1 hour, the plates were washed, then incubated with substrate solution. The substrate reaction was stopped with 1M H_2SO_4, and read as described in section 2.2.14a.

2.2.14e) Hyaluronan Competitive ELISA

High binding flat bottom 96 well plates (Corning Costar, High Wycombe, Bucks.) were coated overnight at room temperature, with hyaluronan 25μg/ml in coating buffer. Plates were then washed in PBS-Tween (4 x 200μl/well) and blocked with 1%BSA in PBS-Tween for 1 hour at room temperature. Samples (BAL fluid was not pre-diluted), standards and biotinylated hyaluronan binding region (kindly provided by Mike Bayliss) diluted in PBS 1%BSA were added together, and incubated overnight at 4°C. After washing in PBS-Tween (4 x 200μl/well), streptavidin-biotinylated HRP complex was added and incubated at 37°C for 30 minutes. The substrate used was either 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in 0.1M citric acid pH 4.0, which was read at 405nm (Fosang et al., 1990), or TMB read at 450nm.

2.2.14f) Calculation of ELISA results

Optical densities for standards, blank wells, and samples were manually entered onto an Apple Macintosh computer and analysed using Assay Zap (Biosoft, Cambridge) software.

2.2.15 Molecular Biology

All reaction steps and reagents were carried out or stored on ice unless otherwise stated.

In order to prevent RNase contamination reagents and pipettes were set aside specifically for RNA work. Sterile disposable plastic ware was used which did not require pre-treatment to inactivate RNases. The laboratory area used was first treated with RNase away (Molecular Bio-Products Inc., San Diego, USA) and disposable gloves were changed frequently.

2.2.15a) RNA extraction

Cells for RNA extraction were either plated onto 6 well tissue culture grade plastic ± 1μg/ml LPS or onto 6 well tissue culture plates pre-coated with hyaluronan (1mg/ml) in monofeed at a cell density of 10^6/ml.

Prior to cell lysis, cell culture supernatant was removed, and 3ml lysis buffer (Trizol, Life Technologies, Paisley, UK) was added to the monolayer, DNA was sheared by vigorous pipetting. Cell homogenates were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. To these samples, 600μl was added, and following a brief shake (by hand), samples were incubated at room temperature for 2 minutes, then centrifuged at 12000 x g for 15 minutes. Following centrifugation two phases were clearly visible. The upper phase was transferred to a sterile RNase-free micro-centrifuge tube, ensuring the interface layer was not disturbed. The lower layer and interface, containing phenol, proteins and DNA was discarded.

2.2.15b) RNA Precipitation

RNA was precipitated with 1.5ml isopropanol, after 10 minutes incubation at room temperature, samples were centrifuged at 12000 x g for 10 minutes. The supernatant was then carefully aspirated, and the RNA pellet was washed once with 2ml 75% ethanol. After centrifuging (7500 x g) for 5 minutes, the pellet was allowed to dry, then re-dissolved in 8µl RNase-free water.

2.2.15c) DNase treatment

Purified RNA was treated with RNase free DNase (10µg/ml, Gibco) to degrade any contaminating DNA. DNase buffer (1µl 10x buffer) was added to the re-dissolved RNA pellet (8µl), with 0.5µl DNase1 (10µg/ml) and 0.5µl placental RNase inhibitor (Life technologies). The RNA was incubated at 37^{o}C for 15 minutes then at 90^{o}C for 10 minutes to inactivate the DNase enzyme.

2.2.15d) cDNA synthesis

DNase treated RNA (10μl) was incubated with reagents described in table 2.3, to give a final volume of 20μl. All PCR reagents were from Life Technologies except dNTPs, which were from Pharmacia Biotechnology (St. Albans, Herts.)

Table 2.3 Reagents for cDNA synthesis

Reagents	Volume (μl)
First Strand Buffer(5 X)	4.0
DTT (100mM)	2.0
Oligo DT (1mg/ml)	1.0
RNase Guard (1U/μl)	1.0
MLV-RT (200U/μl)	1.0
dNTP (10mM)	1.0

This first strand mix was pipetted thoroughly then incubated at 37°C for 60 minutes to allow synthesis of cDNA from RNA, followed by 90°C for 10 minutes, to inactivate reverse transcriptase.

2.2.15e) PCR

cDNA (2μl) was amplified by PCR, using TNFα primers (Stratagene) in the PCR mix outlined below (Table 2.4). Two drops of mineral oil was added to prevent evaporation during the PCR reaction.

Table 2.4 Reagents for PCR

PCR Reagents	Volume(μl)
PCR Buffer* (10 X)	100
dNTP (2mM)	100
Sense Primer (50mM)	20
Antisense Primer (50mM)	20
RNase Free dH_2O	760

* 500mM KCl, 100mM Tris-Cl (pH8.4), 25mM $MgCl_2$

Sense primer; 174 CGGGACGTGGAGCTGGCCGAGGAG 197

Antisense primer; 504 CACCAGCTGGTTATCTCTCAGCTC 528

TNFα primers were synthesised, based on published cDNA sequence in Pennica et al., (1984), and reconstituted in 5mM Tris-HCl, 1mM EDTA.

Controls included PCR mix alone and first strand DNA without Taq polymerase (Life Technologies).

cDNA was also amplified for GAPDH to confirm the presence of RNA and to indicate appropriate loading. The sequence for GAPDH cDNA was obtained from the genebank entry (http://www.ncbi.nih.gov/genebank/query_form.html) and synthesised by MWG Biotech (Milton Keynes,U.K.).

Sense primer; 5'-CCACCCATGGCAAATTCCATGGCA-3'

Antisense primer; 5'-TCTAGACGGCAGGTCAGGTCAACC-3'

GAPDH primers were based on cDNA sequence in Maier et al., 1990.

PCR reactions comprised 5 minutes at 98°C after which point 1μl Taq polymerase was added and the mixture was subject to a 5 minute annealing at 60°C followed by 35 cycles of 1.5 minutes at 72°C, 45 seconds at 94°C, and 45 seconds at 60°C, with a final extension of 10 minutes at 72°C.

Amplified PCR products were diluted in loading buffer (10% glycerol, 10% Ficoll, 0.25% bromophenol blue, 10mM EDTA) and visualised by electrophoresis on 1.2% SeaKem LE Agarose (Flowgen) gels containing 0.5μg/ml ethidium bromide (Sigma), viewed under ultra-violet transillumination. The expected TNFα product was 355bp and GAPDH was 600bp. DNA reference ladder (1kb) was obtained from Life Technologies.

2.2.16 5A4 Preparation

2.2.16a) Hybridoma Culture

5A4 hybridoma cells (a gift from G. Dougherty, Los Angeles), stored in liquid nitrogen, were defrosted rapidly (37°C), washed in warm DMEM and resuspended in DMEM with additives (Penicillin (0.5U/ml), Streptomycin (0.5U/ml), and L-glutamine (2mM). These were then centrifuged and seeded at 0.3 x 10^6/ml in DMEM with additives and 10% foetal calf serum in tissue culture flasks. Cells were split into two flasks after 3 days and were kept at less than 10^6 cells/ml by splitting regularly, until antibody was harvested. To obtain a concentrated antibody supernatant, cells were allowed to proliferate until the medium was exhausted, at which time the cell supernatant was removed, centrifuged (225 x g), to remove cell debris, and stored at -20°C until required.

2.2.16b) 5A4 Purification

5A4 cell culture supernatant was tested for the presence of antibody by flow cytometry (figure 2.5), and then pooled.

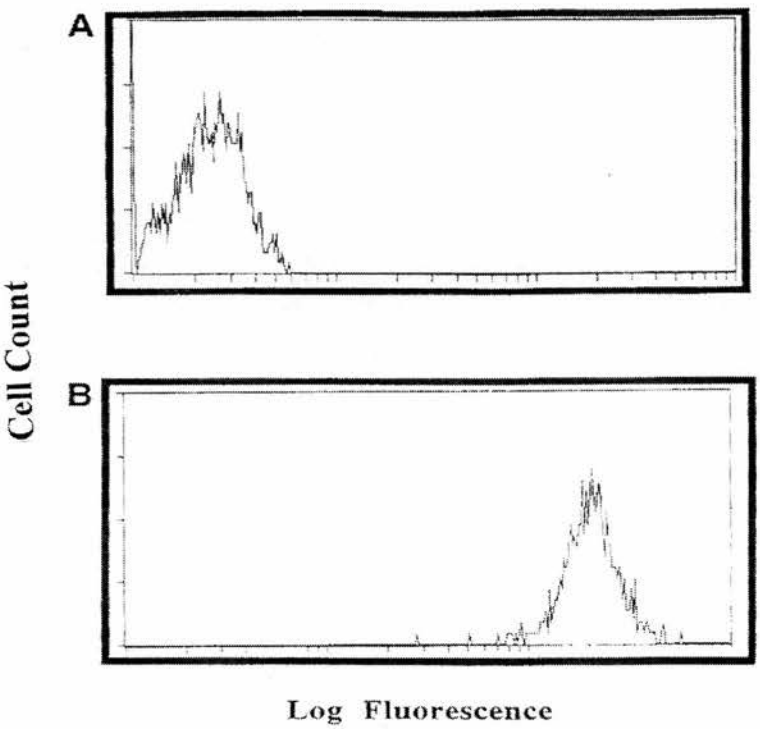


Figure 2.5

5A4 supernatant was tested for the presence of CD44 antibody by using indirect immunofluorescence on peripheral blood monocytes, and flow cytometric analysis. Histograms from one representative experiment are shown, illustrating high levels of CD44 mAb binding to monocytes.

A; negative control.

B; 5A4 supernatant.

Supernatant was concentrated by placing in 76mm dialysis tubing on a tray with Aquicide II (Calbiochem, Nottingham), which served as a desiccant, overnight (4°C). Concentrated 5A4 was then precipitated with an equal volume of saturated ammonium sulphate (4.1M) pH 7.0, overnight at 4°C. This precipitate was resuspended in phosphate buffer (pH 8.0) and dialysed against phosphate buffer pH 8.0 overnight at 4°C. Dialysed 5A4 was adjusted to pH 8.0 with 1M Tris, centrifuged at 895 x g for 15 minutes, and loaded onto a protein-A Sepharose, CL-4B (Pharmacia, St Albans, Herts.) column. Prior to loading the concentrated 5A4 supernatant, the column was regenerated by running 50ml 0.1M Tris/0.5M NaCl, pH 8.5, followed by 50ml 0.1M sodium acetate/0.5M NaCl, then 100ml 0.1M phosphate buffer, pH 8.0. After loading, the column was again washed with 0.1M phosphate buffer (pH 8) and the absorbance of the eluted solution (280nm) was checked using a spectrophotometer (PU8620, Phillips). When eluted fractions contained no detectable protein, the column was eluted with 0.1M citrate buffer, pH 6.5. Eluted fraction were checked for absorbance and the fractions with high absorbances were pooled and dialysed in PBS overnight at 4°C. Purified 5A4 was stored in aliquots at approximately 1mg/ml at-20°C until required.

2.2.16c) 5A4 Purity

5A4 purity was assessed by SDS-PAGE and western blotting (figure 2.6).

Table 2.5 SDS PAGE Reagents

	9% Stacking Gel	9% Separating Gel
STK (stacking gel buffer): 0.5M Tris, pH 6.8	2.5ml	-
SEP (separating gel buffer): 1.5M Tris, pH8.8	-	2.5ml
30% Acrylamide/ 0.8% N'N' methylene bis-acrylamide	3.0ml	3.0ml
20% Sodium Dodecyl Sulphate (SDS)	50µl	50µl
N,N,N'N'-tetramethylethylenediamine (TEMED)	20µl	20µl
10% Ammonium Persulphate	40µl	40µl
dH_20	4.4ml	4.4ml

Sample Buffer (10ml stock): 2.5ml STK, 2ml 20% SDS, 5.5ml 50% (v/v) glycerol, 20µl bromophenol blue (1% in ethanol).

Gels were poured taking care to avoid bubbles and left to polymerise according to the above protocol using the Mini Protean II gel apparatus (Biorad, CA). The samples (in sample buffer) were heated to 95^{o}C for 5 minutes then loaded onto the gel, with 10µl pre-boiled molecular weight markers (Biorad CA) in a separate lane. Proteins were separated at 30mA constant current for 2 hours (or until dye front reached the bottom of the gel) using 25mM Tris/192mM glycine 0.1% SDS (pH 8.3) as the electrophoresis buffer.

Gels were either stained for total protein with 0.25% (w/v) Coomassie Brilliant Blue; with 10% glacial acetic acid, followed by destaining in 40% methanol/10% glacial acetic acid, or blotted onto nitro-cellulose membranes (Hybond C, Amersham, UK) using Mini Protean II Transfer apparatus (Biorad, CA) with transfer buffer (25mM Tris/192mM glycine/ 20% methanol) for 4 hours at 400mA.

Purified 5A4 was titrated for binding by flow cytometry (figure 2.7).

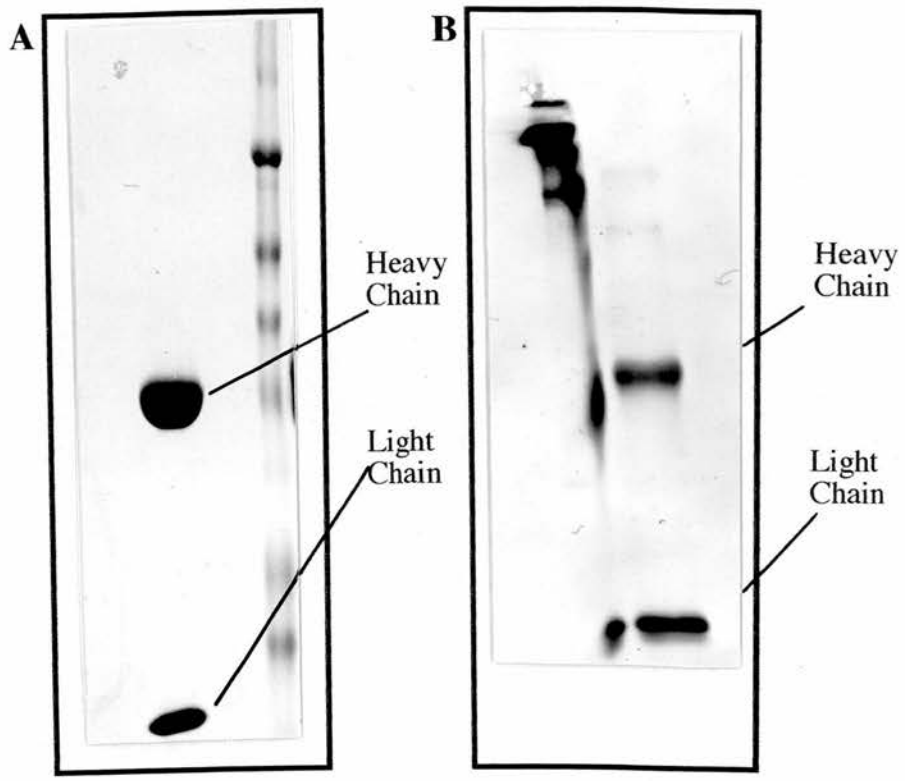


Figure 2.6

Purified 5A4 was assessed for purity by SDS-PAGE and western blotting. Photographs of representative **A)** acrylamide gel showing reduced 5A4 and molecular weight markers. **B)** blot showing the presence of antibody in samples of purified 5A4, (non-reduced and reduced).

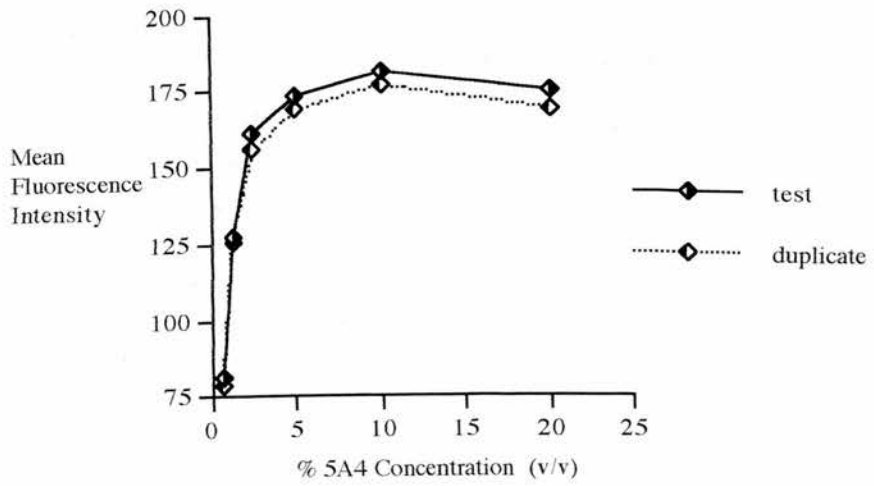


Figure 2.7

Purified 5A4 monoclonal antibody binding to monocytes was assessed by titration. Mean fluorescence, as determined by flow cytometric analysis, was used to assess saturating concentrations of binding. Results from one representative experiment. Purified 5A4 was found to bind optimally at 5% (1:20 dilution).

2.2.16d) Western Blotting

After transferring proteins onto nitro-cellulose membranes, blots were blocked in TBS with 0.5% Tween 20 for 1 hour. Blots were then incubated with goat-anti-mouse HRP (1:1000) for 30 minutes. After washing in TBS with 0.5% Tween 20, staining was visualised using DAB, 50μg/ml in TBS pH 7.6 with 10μl 30% H_2O_2, this substrate reaction was stopped with water.

2.2.17 Assessment of cell viability and numbers

Cells suspended in either PBS (peripheral blood monocytes and alveolar macrophages) or in medium (5A4 hybridoma cells) were diluted 1:2 with 0.2% trypan blue (Sigma) and 10μl was carefully loaded onto a haemocytometer. Cell viability was assessed through the exclusion of trypan blue, and cell numbers were assessed by counting 2 x 16 large squares, which gave a cell number of n x 10^4/ml.

2.2.18 Statistical analyses

All statistical analyses were performed using Statview for Apple Macintosh. Where appropriate (normally distributed data) the two-tailed paired Student's T-test was used. For non-parametric analysis (BAL samples, chapter 5), the Mann Whitney-U test was used. Statistical significance was attained with p-values of less than 0.05.

Chapter 3

3.1 Introduction

3.1.1 Surface Antigen Expression

Myeloid cells, like other leukocytes, possess cell surface antigens which direct cell to cell contact and the interactions with soluble pro-inflammatory mediators, functions that are necessary for immunological homeostasis. Expression of surface antigens may be altered upon activation or upon maturation. For example, during myeloid cell differentiation, expression of CD14 (receptor for LPS), which is not expressed on monoblasts (Zeigler-Heitbrock et al., 1993), increases with maturation and is strongly expressed on monocytes (Goyert et al., 1988). Monocyte-derived tissue macrophages may maintain CD14 expression e.g., peritoneal macrophages (Andreesen 1990), or may down-regulate expression e.g., alveolar macrophages (Barbosa et al., 1991). Surface molecules may thus reflect the state of activation of cells, and serve as a trigger of inflammatory events.

Altered surface antigen expression in alveolar macrophages has been observed in lung carcinoma (Barbosa et al., 1991) and in the chronic interstitial lung disease, pulmonary sarcoidosis (Hoogesteden 1989). More recently altered surface antigen expression (CD11a, CD11b, and CD11c) was shown to be increased in alveolar macrophages from patients undergoing cardiac surgery (Tsuchida et al., 1997). Differences in antigen expression may be caused by local factors such as changes in surrounding cell populations and their activation states (Striz et al., 1993), or by production of inflammatory mediators (Kasinrerk et al., 1993, Liao and Simon 1994, Kruger et al., 1996). In fact, cytokine production in sarcoidosis has been linked with

altered alveolar macrophage expression of surface antigens such as CD14 (Zheng et al., 1995).

3.1.2 Alveolar Macrophage Autofluorescence

The search for markers to identify people at risk of ARDS is intense. However, alveolar macrophages, which are the main immunocompetent cells in the lung, reside in a unique environment in which they are exposed to both blood borne mediators and inhaled immunogens. This exposure to inhaled substances such as tobacco smoke may contribute to the high autofluorescence observed in alveolar macrophages (Skold et al., 1989). Autofluorescence in macrophages from non-smokers may be caused by the storage of a variety of flavoproteins, and exogenous pigments (Benson et al., 1979, Lenhert et al., 1986) and is induced after excitation by argon lasers (the most common lasers in flow cytometers) which emit light at a wavelength of 488nm (Havenith et al., 1993). The resulting autofluorescence at 541nm and 580nm (Viksman et al., 1994) overlaps with the fluorescence emitted by fluorophores such as fluorescein isothiocyanate and R-phycoerythrin, which emit at 480 to 580nm and 540 to 640nm respectively (Edelson et al., 1985).

In this chapter, macrophage surface antigen expression was investigated as a possible indicator of cell activation, particularly in the pathogenesis of ARDS. Methods employed to overcome the problem of autofluorescence are outlined, during attempts to surface phenotype alveolar macrophages, obtained by bronchoalveolar lavages from both normal healthy volunteers, and patients at risk of ARDS.

3.2. Results

3.2.1 Monocyte-Macrophage Maturation

Monocyte differentiation into macrophages is associated with alterations in morphology; figure 3.1 shows the cell size of monocytes compared with figure 3.2 which shows macrophages derived from monocytes cultured for 5 days *in vitro*.

Monocytes and monocyte-derived macrophages were phenotyped using a comprehensive panel of well-defined monoclonal antibodies, to provide a phenotypic reference for flow cytometric analysis of macrophages from BAL (figure 3.3).

Upon *in vitro* maturation the expression of CD11b decreased whereas expression of CD11a remained relatively high, freshly isolated monocytes expressed high levels of both CD11a and CD11b.

MHC class II and CD14 were chosen for preliminary studies of alveolar macrophages for two reasons. Both molecules are relatively well established macrophage markers and show regulated expression. The latter suggestion is supported by data in figure 3.3, which shows increased class II expression during *in vitro* culture whereas CD14 expression is decreased.

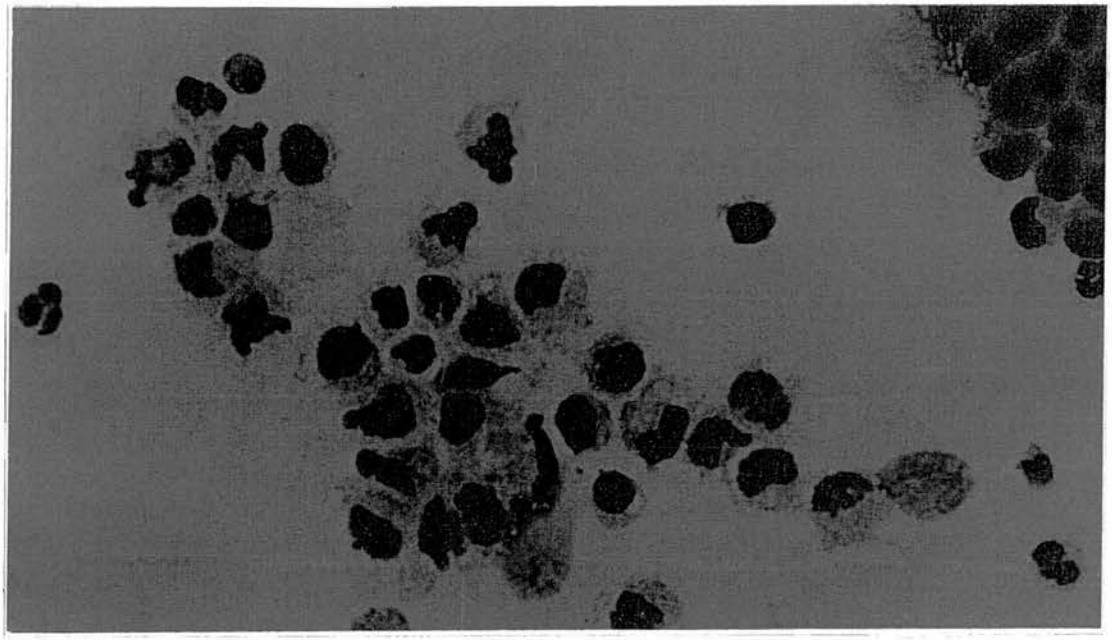


Figure 3.1

Photomicrograph showing freshly isolated monocytes, photographed using a x 40 objective.

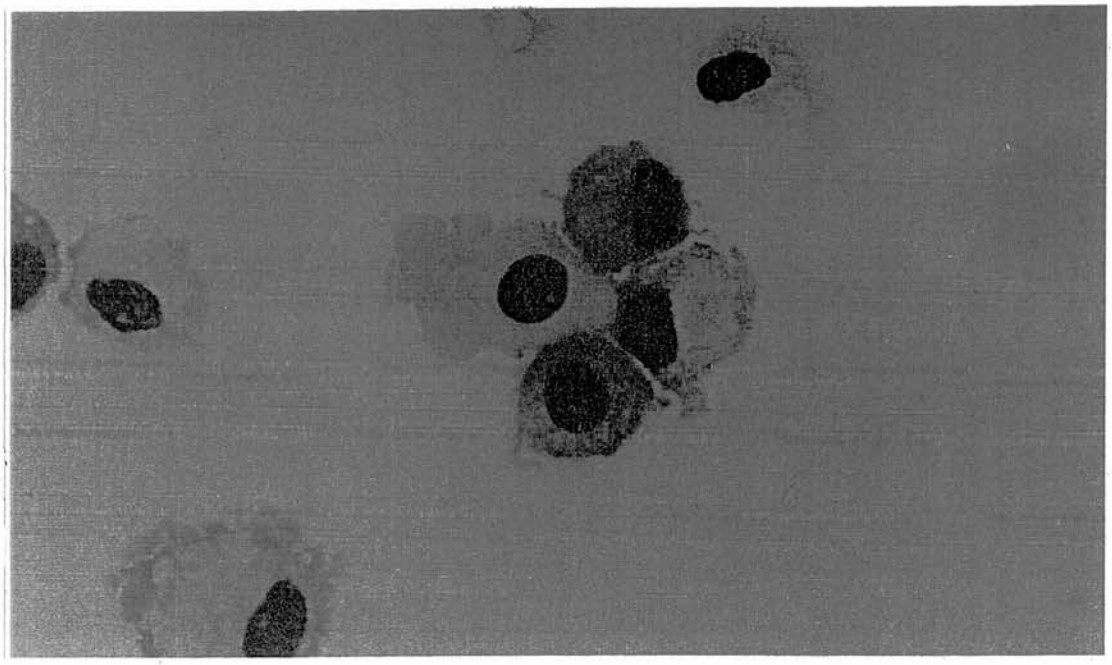


Figure 3.2

Photomicrograph showing monocyte-derived macrophages, photographed using a x 40 objective.

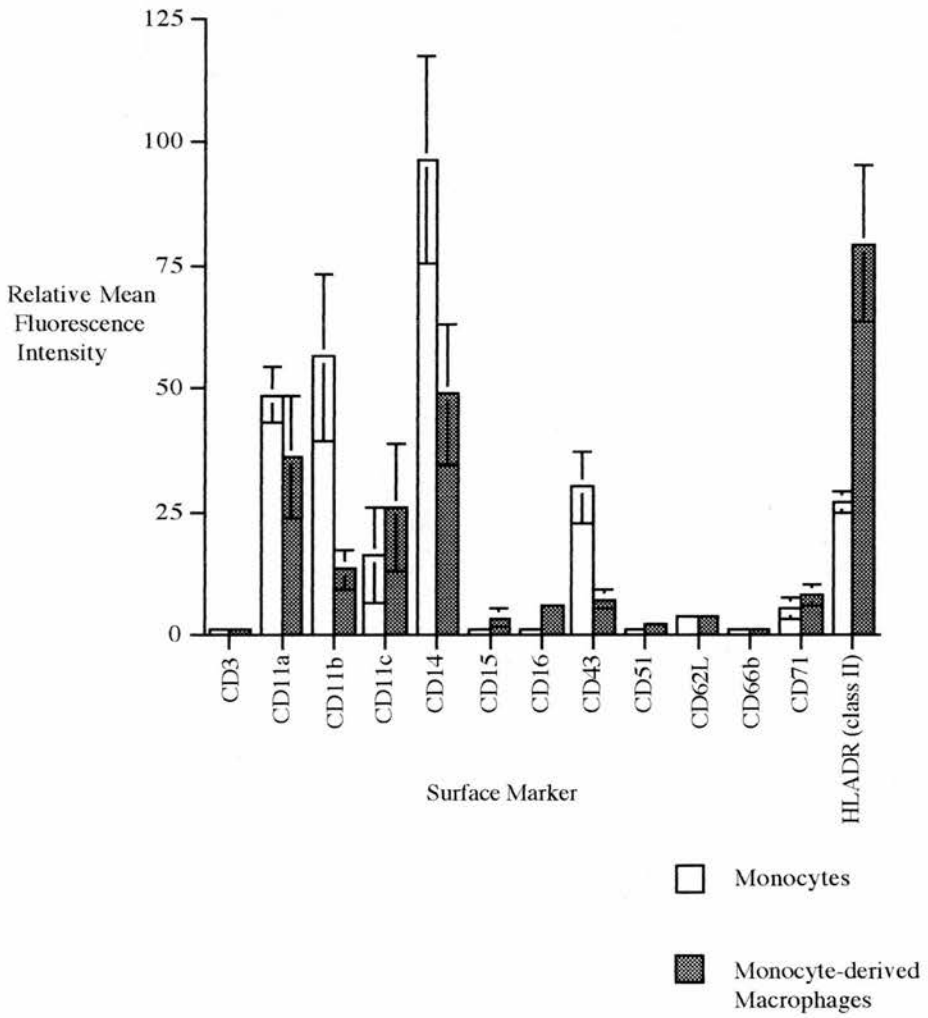


Figure 3.3

Monocytes and 6 day monocyte-derived macrophages from the same donors were stained for surface markers by indirect immunofluorescence, and analysed by flow cytometry. Mean fluorescence intensity is expressed relative to that recorded for negative controls (n=7).

3.2.2 Cell bound ELISA

Flow cytometric analysis of alveolar macrophages (particularly from smokers) revealed high levels of autofluorescence (not shown). I therefore investigated different methods to overcome this problem. An alternative method to flow cytometry for quantitation of surface receptor expression was the use of an enzyme-linked marker in a colorimetric system which was implemented thus avoiding fluorescence based assessments. This system was first validated with peripheral blood monocytes. Figure 3.4 shows the optical densities for CD11a, CD14, and MHC Class II, relative to negative controls, compared with relative mean fluorescence intensity in cells from the same donors. When the substrate reaction (β-galactosidase) was performed at room temperature, very low optical densities were observed (figure 3.4). Although increasing the substrate reaction temperature to 37°C increased optical densities, the observed values did not correlate well with flow cytometric analysis. One possibility was that surface marker expression was reduced as a consequence of using adherent cells, when compared with non-adherent cells used in flow cytometric analysis. To address this possibility, subsequent assays were performed with cells in suspension (figure 3.4). However, the results obtained from three experiments with this modification were not reproducible (not shown). In contrast to flow cytometry, which provides an estimate of the numbers of surface receptors per cell, the ELISA gives a measure of antibody reactivity for the total cell population present, and as a consequence is very dependent on the absolute cell number.

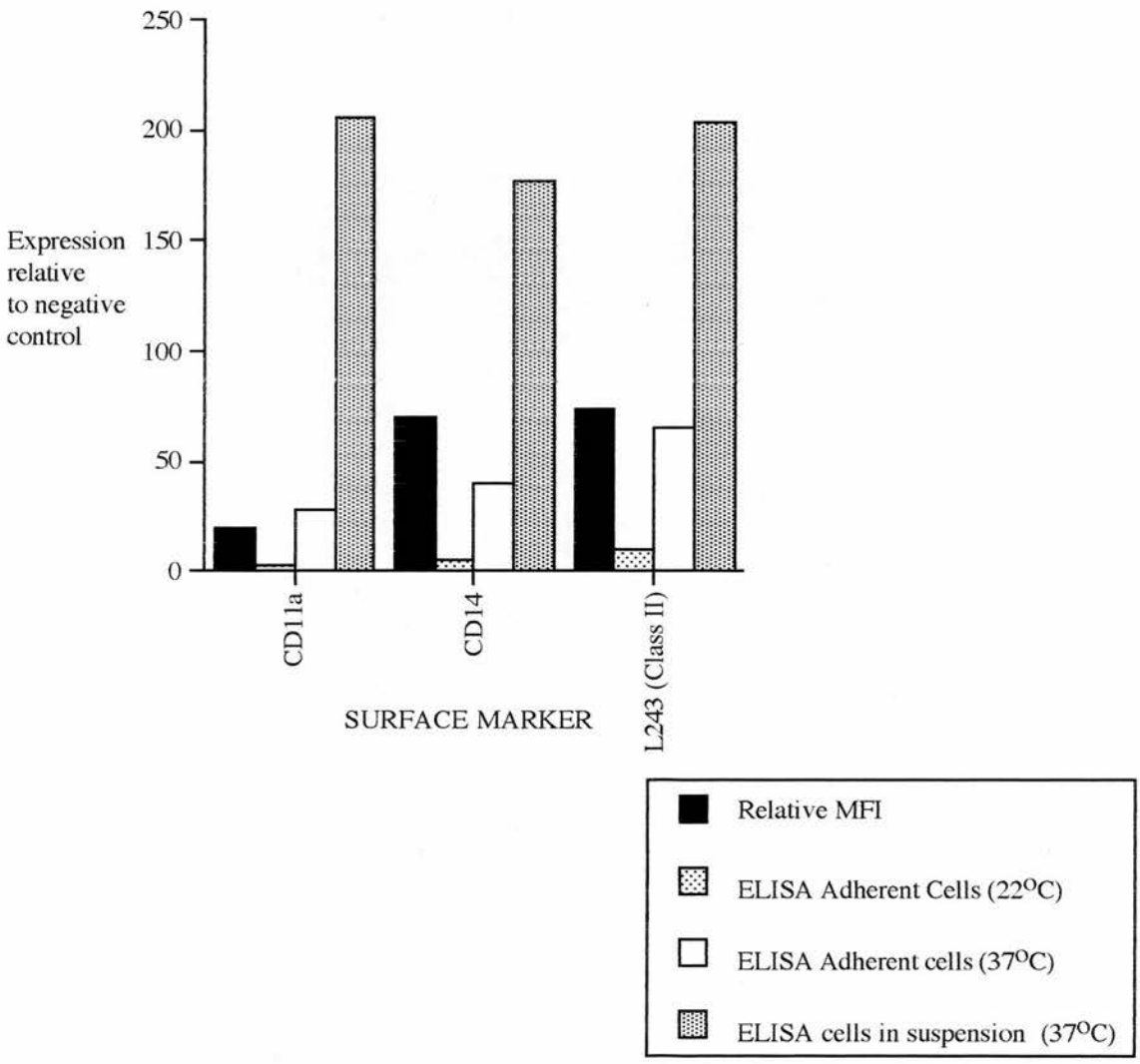


Figure 3.4

Monocyte cell surface markers expression was quantified using a cell bound ELISA technique (n=3), and compared with the relative mean fluorescence obtained by flow cytometric analysis of monocytes from the same donors.

Values from cell bound ELISA are represented by the optical density relative to negative controls (cells incubated with irrelevant IgG, MOPC).

Values from flow cytometric analysis (relative mean fluorescence intensity) are expressed relative to the recorded negative control (MOPC).

3.2.3 Quenching Autofluorescence

As an alternative approach, Hallden et al., (1991) described a method using crystal violet to "quench" intrinsic cellular autofluorescence. Using this quenching method, I compared flow cytometric determination of levels of CD11b, CD11c, and CD14 on quenched monocytes, and untreated monocytes. Initial experiments suggested that mean fluorescence intensity was not significantly affected by quenching (figure 3.5a). I therefore extended the analysis to use alveolar macrophages from normal volunteers, obtained by bronchoalveolar lavage (BAL). Crystal violet quenching significantly reduced the fluorescence emitted from alveolar macrophages stained for CD11b, CD11c and CD14 (figure 3.5b, $p<0.05$). Relative fluorescence on alveolar macrophages for these surface antigens appeared to be lower than the corresponding values on monocytes (figure 3.5c). However, the results obtained using the quenching method were found to be somewhat variable, which may have been due in part, to the formation of precipitates in the crystal violet solution. Although heating, followed by filtering dissolved solutions, batch variations appeared to be unavoidable.

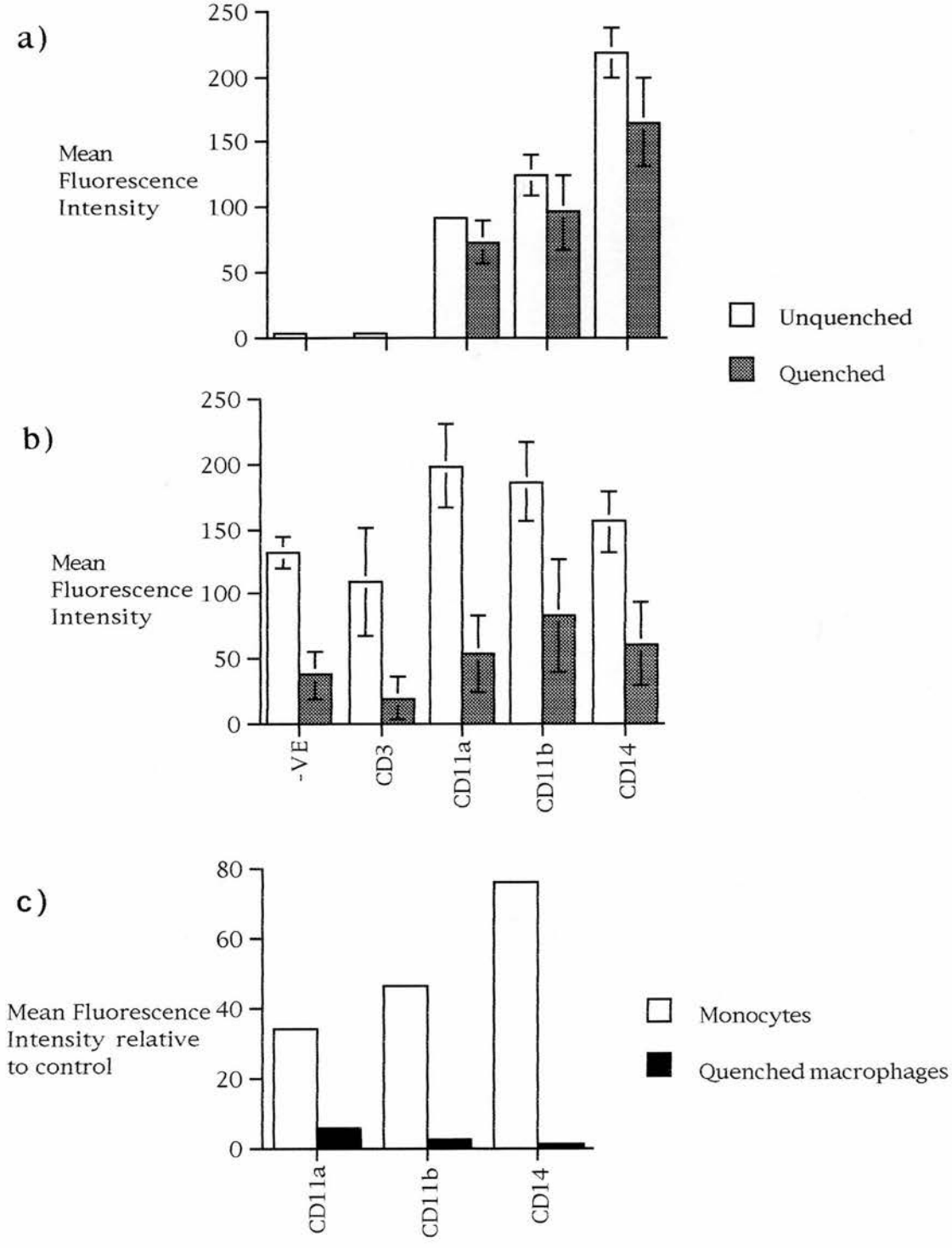


Figure 3.5

Monocytes (a) and alveolar macrophages (b) were "quenched" with crystal violet prior to surface phenotyping by flow cytometry. Mean fluorescence intensities, relative to values obtained with negative control were compared (c), n=3.

3.2.4 Tandem Dye Flow Cytometry

To overcome problems with autofluorescence, an alternative method for phenotyping alveolar macrophages employing new tandem fluorescent dyes, was assessed. These dyes consist of R-phycoerythrin (which can be excited with an argon laser at 488nm) linked to Cy5, which has a peak emission at 670nm and is thus outside the autofluorescent range. Using the indirect immunofluorescence technique described in chapter 2, two such products were tested; Quantum Red (Sigma) and TriColour (Bradsure Biologicals). After initial titrations using alveolar macrophages from normal volunteers the BAL cells from a trauma victim at risk of ARDS were phenotyped using the two products (figure 3.6). A more complete experiment would have included the staining of monocytes using these two products. However, a further experiment on trauma patient alveolar macrophages, using a panel of surface antigens with TriColour resulted in high fluorescence 1 and 2 (the green and red channels) but low fluorescence 3 (data not shown). These inconsistencies in experimental results were considered to be a significant problem when dealing with valuable patient samples.

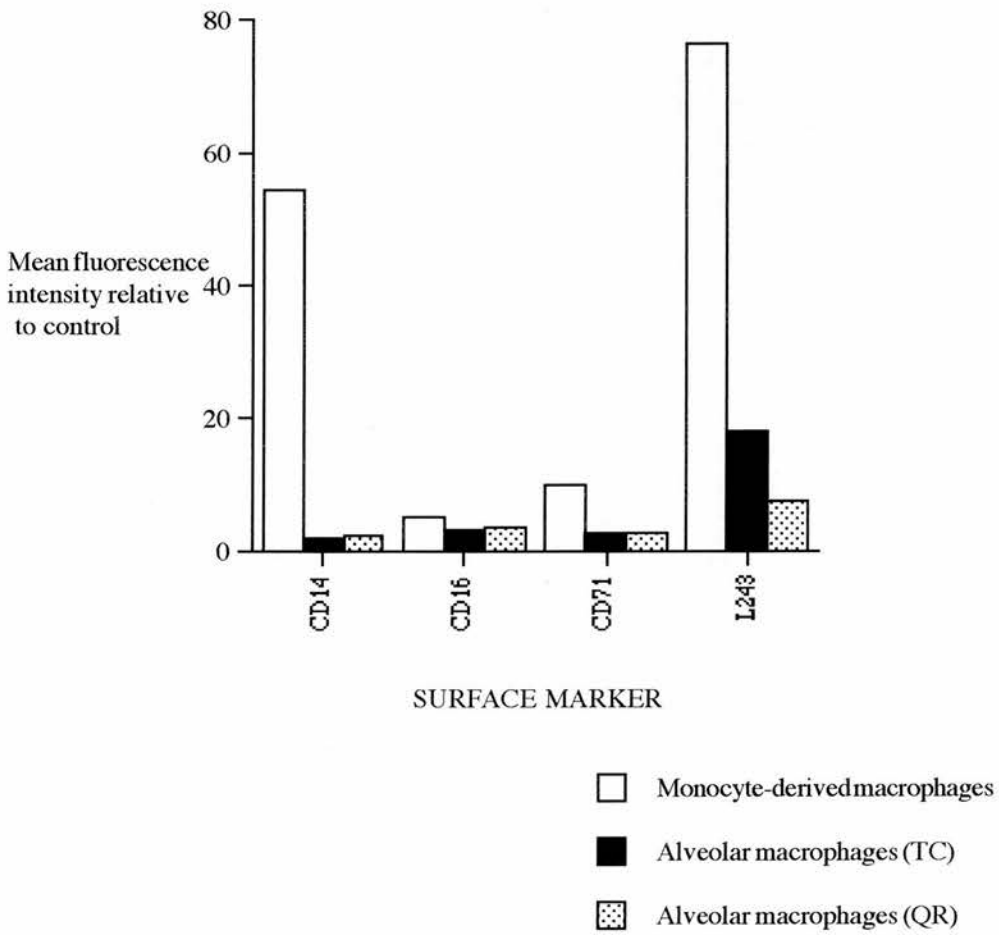


Figure 3.6

Cell surface markers on alveolar macrophages taken from a trauma victim at risk of ARDS were assessed by flow cytometry, using the tandem dyes TriColour (TC) and Quantum Red (QR), and were compared with the relative expression of monocyte-derived macrophages, stained by indirect immunofluorescence.

3.3 Discussion

Monocyte to macrophage maturation *in vitro* is characterised by a decrease in peroxidase activity, an increase in cell size and changes in surface antigen expression (Johnson et al., 1977). These alterations reflect the different roles myeloid cells play throughout their differentiation, e.g., CD16 (FcγRIII, involved in phagocytosis) not expressed on monoblasts, is weakly expressed on monocytes, and is strongly expressed on alveolar macrophages and other tissue macrophages (Zeigler-Heitbrock 1993). The induction of CD16 in culture-derived macrophages is thus indicative of their maturation state. Similarly the decreased expression of CD11b, which has previously been reported as a marker for monocytes and granulocytes (Hogg, 1987) indicates the *in vitro* maturation of these cells.

The increased HLA-DR expression noted in monocyte-derived macrophages and in tissue macrophages (Andreesen et al., 1990, Krombach et al., 1996) may reflect the increased functional requirements within inflammatory sites, i.e., processing and presentation of antigens, especially within the lung where inhaled immunogens provide a requirement for immunological competence and co-operation.

Interestingly, expression of CD14 was down-regulated upon *in vitro* culture, this confirms previous studies of cultured monocytes (Kaplan et al., 1982, Zeigler-Heitbrock et al., 1993) and indicates that monocyte-derived macrophages are comparable with alveolar macrophages in this respect. However CD14 expression in monocytes has also been reported to increase upon *in vitro* culture (Gessani et al., 1993, Hopkins et al., 1995, Audran et al., 1996). These differences may be explained by the presence of serum factors in the culture media, as increased CD14 expression

occurred in studies where foetal calf serum was substituted for human serum, and Hopkins et al., (1995) showed that the presence of foetal calf serum enhanced LPS-induced up-regulation of CD14 in alveolar macrophages. Alternatively, the presence of contaminating LPS may result in the up-regulation of CD14 expression (Hopkins et al., 1995, Landmann et al., 1996). The presence of contaminating lymphocytes and associated lymphokine production, may also influence monocyte expression of CD14 *in vitro*, GM-CSF has been shown to decrease CD14 expression in monocytes and monocyte-derived macrophages (Kruger et al., 1996).

Increased CD71 (transferrin receptor) expression was also observed during monocyte culture *in vitro*, this receptor is involved in the control of iron supply to the cell, through the binding of the major iron carrier protein, transferrin. The up-regulated expression of CD71 upon *in vitro* culture of monocytes is well documented (Hirata 1986, Andreesen et al., 1990, Bennet et al., 1992), and is probably associated with increased bactericidal activities (Johnston 1978, Alford et al., 1991), again highlighting the altered requirements for expression of surface molecules on maturation from monocytes to macrophages.

The expression of surface antigens, is quantitatively analysed by the use of flow cytometry which records the relative expression on thousands of cells. However autofluorescence seen in alveolar macrophages presents problems for quantitation of surface receptor expression when using methods that employ the use of fluorescent markers (Edelson et al., 1985). Although cell bound ELISA represents an alternative method avoiding fluorescent detection, the absolute cell numbers affect the final substrate reaction and consequently may give rise to inaccurate results, especially when non-adherent cells are used.

An alternative approach is to quench intrinsic autofluorescence through the use of crystal violet. Results presented in this chapter show significantly decreased fluorescence following crystal violet treatment in normal alveolar macrophages. Using this technique, the relative expression of all antigens examined (CD14, CD11a, CD11b) was found to be lower than that on monocytes and monocyte-derived macrophages.

These data are consistent with previous studies which showed that these antigens are down regulated in alveolar macrophages from normal donors (Barbosa et al., 1991, Perez-Arellano et al., 1993, Viksman et al., 1994, Prieto et al., 1994). The low levels of expression of adhesion molecules found in lavage macrophages may reflect the low adhesive state of cells that are removed from the alveoli. Tsuchida et al., (1997) showed CD11a, CD11b, and CD11c expression was increased on alveolar macrophages as a result of cardiopulmonary bypass during cardiac surgery. This increase in adhesion molecule expression may have indicated a primed state in these cells *in vivo*, as an increase in production of TNFα and IL-8 was seen in these otherwise unstimulated macrophages *in vitro*. However the up-regulation of these adhesion molecules was not accompanied by any impairment of lung function.

Analysis of alveolar macrophages from one trauma patient using tandem fluorescent dyes, suggested low levels of expression of HLA-DR. Although further analyses of patient samples would be required to test the possibility that trauma resulted in reduced expression of HLA-DR. As alveolar macrophages are in constant contact with inhaled immunogens a high expression of MHC class II molecules might be

expected, in fact high levels of HLA-DR have previously been reported in alveolar macrophages (Viksman et al., 1994).

Repeated experiments using the tandem dyes Quantum Red and TriColour resulted in fluorescence in the green and red channels emitted by the autofluorescence and phycoerythrin but no visible fluorescence in the third channel, suggesting that Cy5 excitation was either not being emitted, or was emitted but not detected. This lack of signal in the third channel had previously occurred using TriColour and a replacement batch was used in the experiment where fluorescence was detected. Although it is possible that technical problems may have occurred within the flow cytometer hardware, the problems associated with the use of these tandem dyes suggested that the Cy5 conjugate may have been unstable.

The results presented in this chapter confirm that macrophages upon maturation from monocytes *in vitro* undergo morphological and phenotypic changes, which can be easily assessed. These changes, including decreased peroxides activity, and increased expression of CD16, CD71, and MHC class II molecules, reflect the changes in cell function, and show some parallels with tissue macrophages. Phenotypical analyses of alveolar macrophages, using flow cytometric analysis (figures 3.5 and 3.6) indicated low levels of surface expression of CD11a, CD11b, CD14, HLA-DR and CD71, but methodological problems and the small numbers of cells recovered from patients at risk of ARDS (as these patients cannot be subjected to aggressive lavage techniques) hindered the phenotypical analysis of alveolar macrophages. In addition the frequency of trauma samples decreased significantly

with the introduction of car safety features such as the drivers airbag, in addition to improved resuscitation methods and more efficient ambulance response times.

CHAPTER 4

4.1 Introduction

4.1.1 Cellular responses to ECM

Within the tissue microenvironment, ECM composition has an important influence over various aspects of cell function. During morphogenesis it has been proposed that the ECM environment provides signals regulating the migration and proliferation of cells (reviewed by Hynes and Lander 1992). Tissue remodelling, both under hormonal control (e.g. milk gland formation) and during angiogenesis is also profoundly influenced by the organisation of ECM components (reviewed in Roskelley et al., 1995, Gailit and Clark 1994).

The extent to which ECM molecules exert their influences over cell motility and proliferation is indicated in numerous *in vitro* studies (Clarke et al., 1982, Brown et al., 1993, Klempe et al., 1994, Peck and Isacke 1996) (Clarke et al., 1997, Ohtaka et al., 1996, Kubota et al., 1988, Elstad et al., 1987, Adamson and Young 1996).

4.1.2 ECM influences in inflammation

During inflammatory responses, leukocyte migration is essential for efficient cell recruitment in response to inciting stimuli and for effective microbicidal and repair mechanisms. Extracellular matrix components may exert influences on the initial stages of cell recruitment as hyaluronan has been shown to mediate rolling in lymphocytes (DeGrendele et al., 1996, Clarke 1996).

It is now clear that cellular responses to cytokines may be altered during inflammation due to the influences of ECM components present in the inflammatory milieu (Loike et al., 1995, Xu and Clark 1996). Conversely, cellular responses to ECM molecules may be modulated by inflammatory cytokines, as many receptors for ECM molecules are activated or modulated in response to cytokines and chemokines (Jiang et al., 1992, Lundahl et al., 1996). Cytokines may also influence leukocyte responses indirectly by modifying the composition of ECM (Pettipher 1986, Wahl et al., 1990, Maniscalco et al., 1994). In addition, degradative enzymes such as matrix metalloproteases, may generate peptides with altered chemotactic properties (Doherty et al., 1990).

Thus, the composition of extracellular matrices can differentially affect the ability of cells to migrate, proliferate, and become activated. Such wide-ranging responses provide a sensitive mechanism for regulation during inflammation and repair.

4.1.3 ECM and secretion

As previously discussed (Chapter 1) monocytes and macrophages are effective secretory cells, and various ECM components have been shown to influence cytokine production. As adherence has been shown to induce monocyte expression of cytokines (Haskill et al., 1988), the nature of monocyte adherence may be important in the production of a monocyte pro-inflammatory cytokine profile. Additionally, changes in ECM composition during inflammation may influence the progression of inflammatory disease. The possibility of ECM composition affecting monocyte cytokine production was investigated.

The ability of macrophages to release the cytokines TNFα, IL-1β, IL-8 and TGFβ, in response to adherence to different ECM substrates was examined. Initial experiments used alveolar macrophages from bronchoalveolar lavage fluid from both trauma patients and normal healthy volunteers. However poor cell yields and variable macrophage viabilities were limiting for mechanistic studies. I therefore developed a more reliable system using peripheral blood-derived monocytes as a model for macrophages, which possess many features characteristic of macrophages, including production of cytokines. In comparison with alveolar macrophages, these cells are more readily purified, with better yields and high viabilities (>50% and 90% respectively). Considering the well-characterised plasticity of monocyte function both *in vivo* and *in vitro*, these cells represent a useful model for studying responses to ECM components.

4.2 Results

4.2.1 ECM Effects on Monocyte Cytokine Release

In order to establish whether different ECM components influenced monocyte cytokine production, monocytes were adhered to tissue culture plastic coated with various different ECM molecules hyaluronan (1mg/ml), heparan sulphate (10μg/ml), vitronectin (10μg/ml) and fibronectin (10μg/ml). Cell supernatants were removed at 4 hours, 8 hours and 24 hours, and were assayed by ELISA for the presence of pro-inflammatory cytokines TNFα, IL-1β, IL-8, and TGFβ. LPS stimulation was used to demonstrate that monocytes were capable of cytokine production, and monocytes adherent to plastic alone represented a negative control. The release of TNFα in response to these ECM substrates is shown in figure 4.1. Significant TNFα release occurred on hyaluronan ($p=0.032$) and in the presence of 1μg/ml LPS ($p=0.003$) when compared to plastic control at 4 hours, and although TNFα release from monocytes on hyaluronan decreased with time, levels remained significantly elevated when compared to plastic alone at both 8 and 24 hours. As there was no significant release of TNFα from monocytes adhered to heparan sulphate, vitronectin, or fibronectin this suggests that the TNFα release from monocytes adhered to hyaluronan was specific. In contrast to the release of TNFα seen upon adhesion to hyaluronan, significant release of IL-1 occurred only at 4 hours on hyaluronan when compared to plastic ($p=0.03$). LPS induced IL-1 release at both 4 and 8 hours, but less IL-1 release was seen at 24 hours. These data confirm the early release nature of these cytokines.

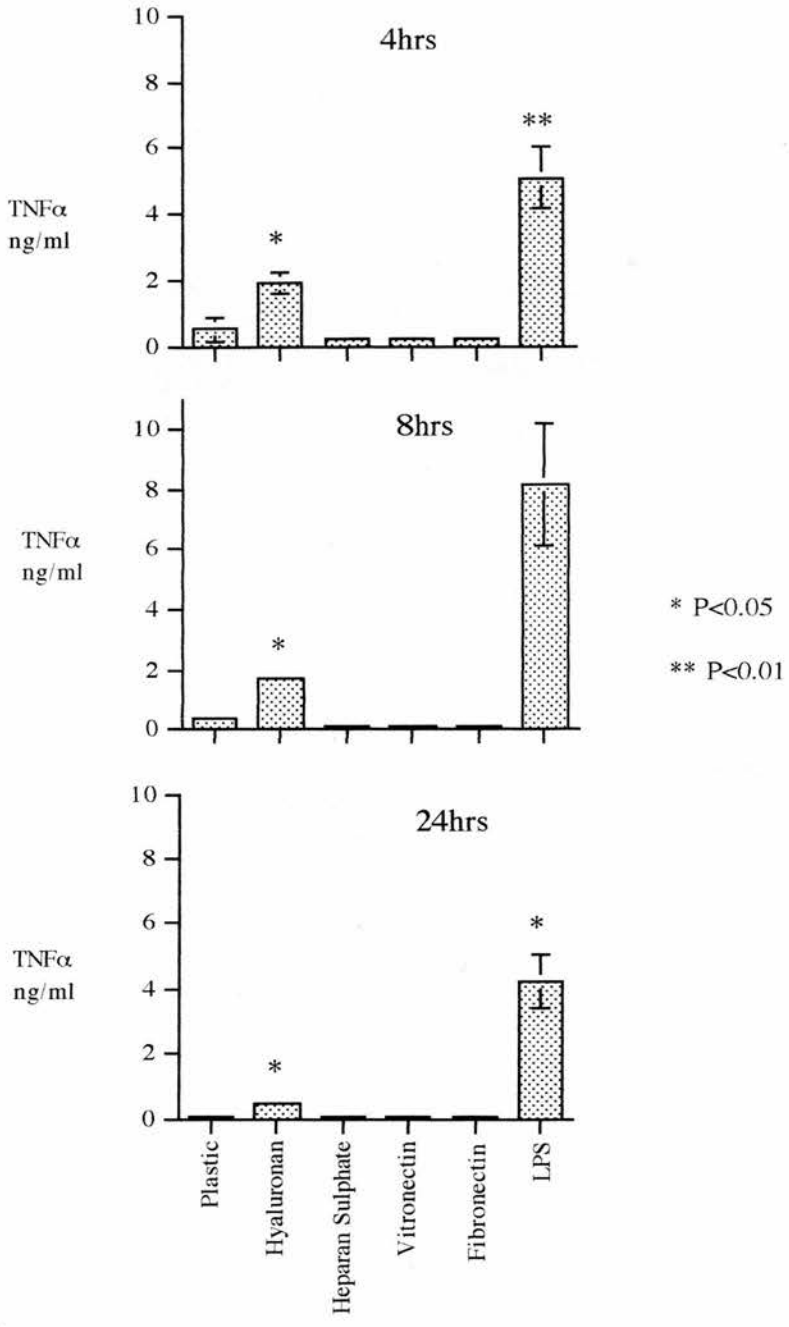


Figure 4.1

Adherence separated monocytes were added to tissue culture plates pre-coated with different ECM substrates; hyaluronan (1mg/ml), heparan sulphate (10μg/ml), vitronectin (10μg/ml), fibronectin (10μg/ml), or onto plastic ± 1μg/ml LPS. TNFα release was assessed by ELISA. Monocytes on hyaluronan release significantly more TNFα than monocytes on control (plastic) substrate ($p < 0.05$). LPS also stimulates cells to release TNFα when compared to plastic ($p < 0.05$). At these time points none of the other substrates tested induced significant TNFα release, n=4. Error bars represent standard error of the mean (SEM), where not visible SEM is small.

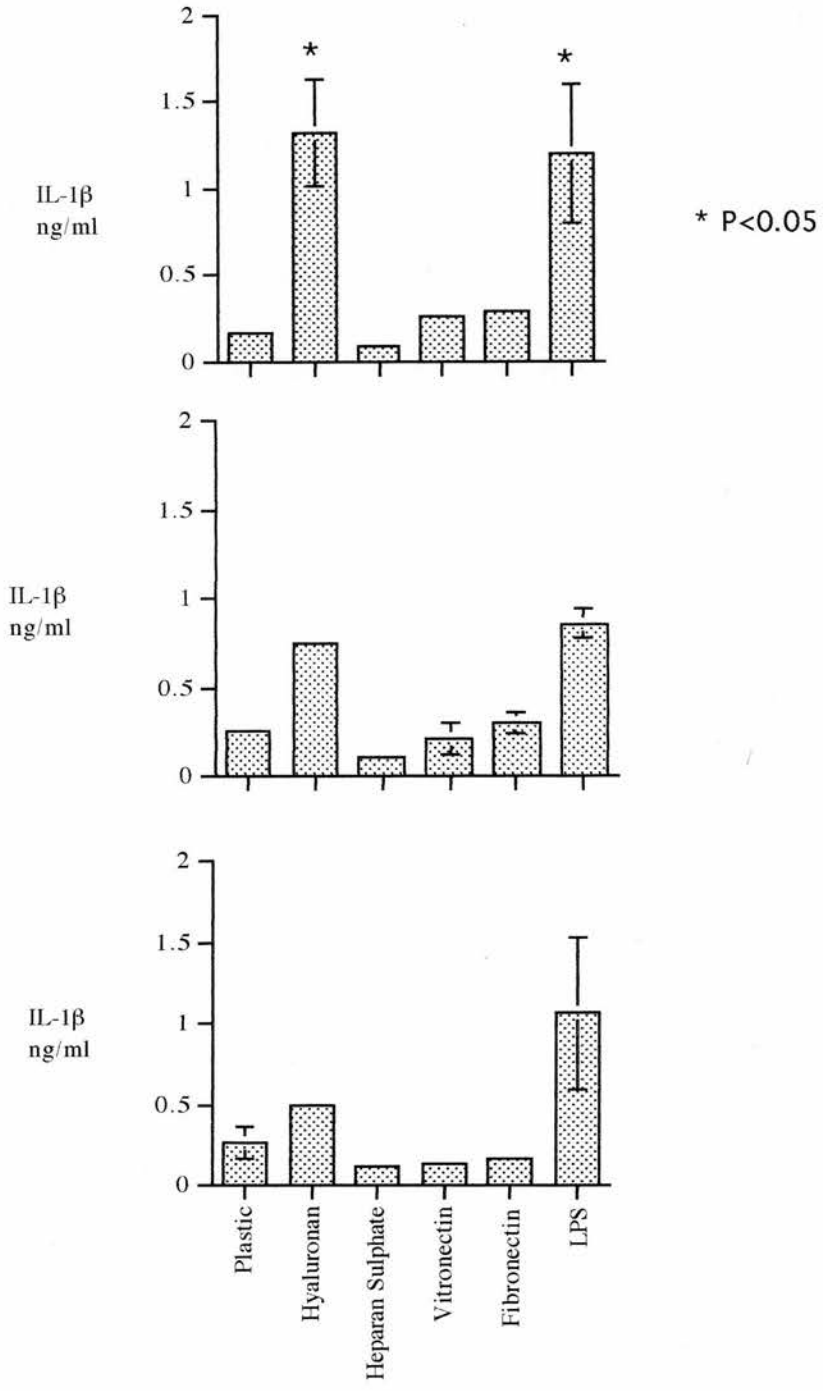


Figure 4.2

Monocytes were adhered to different ECM substrates; hyaluronan (1mg/ml), heparan sulphate (10μg/ml), vitronectin (10μg/ml), fibronectin (10μg/ml), or onto plastic ± 1μg/ml LPS. IL-1β release was measured by ELISA. Monocytes stimulated with LPS, or adherent to hyaluronan release significantly more IL-1β than monocytes on control (plastic) substrate ($p < 0.05$) at 4 hours, n=4. Error bars represent standard error of the mean (SEM), where not visible SEM is small.

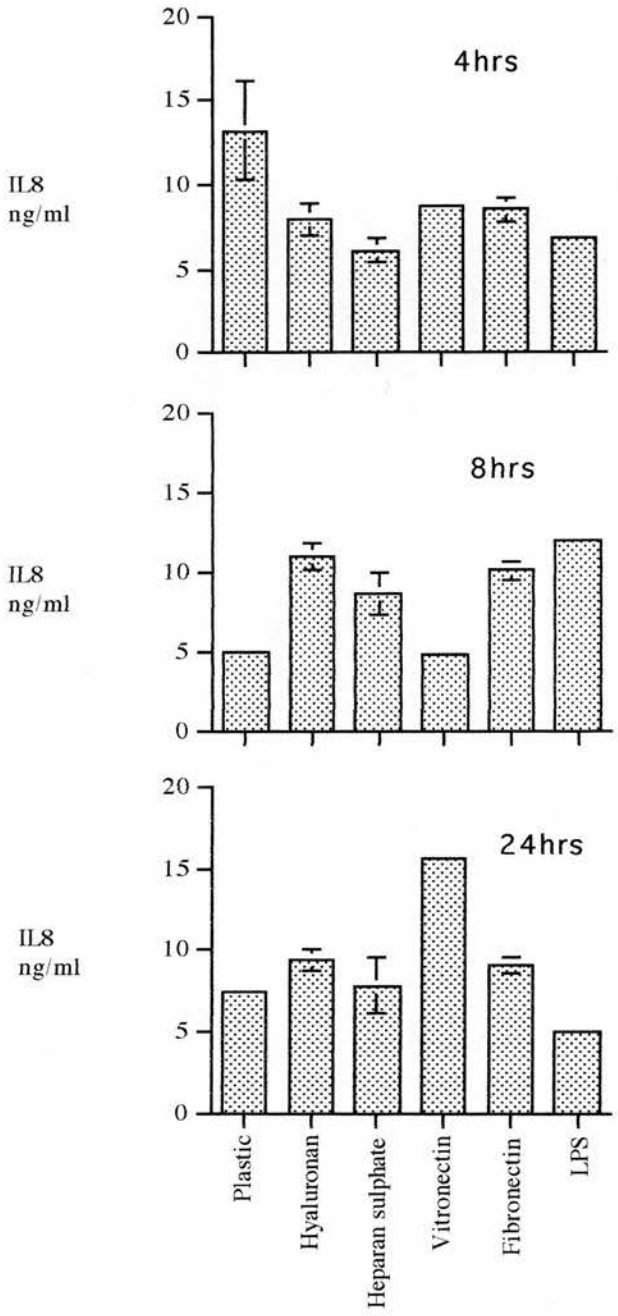


Figure 4.3

Effect of different ECM substrates on the release of IL-8 from adherence separated monocytes (n=4), IL-8 was assessed by ELISA. At the times investigated there were no significant differences in IL-8 release from monocytes on different substrates. Error bars represent standard error of the mean (SEM), where not visible SEM is small.

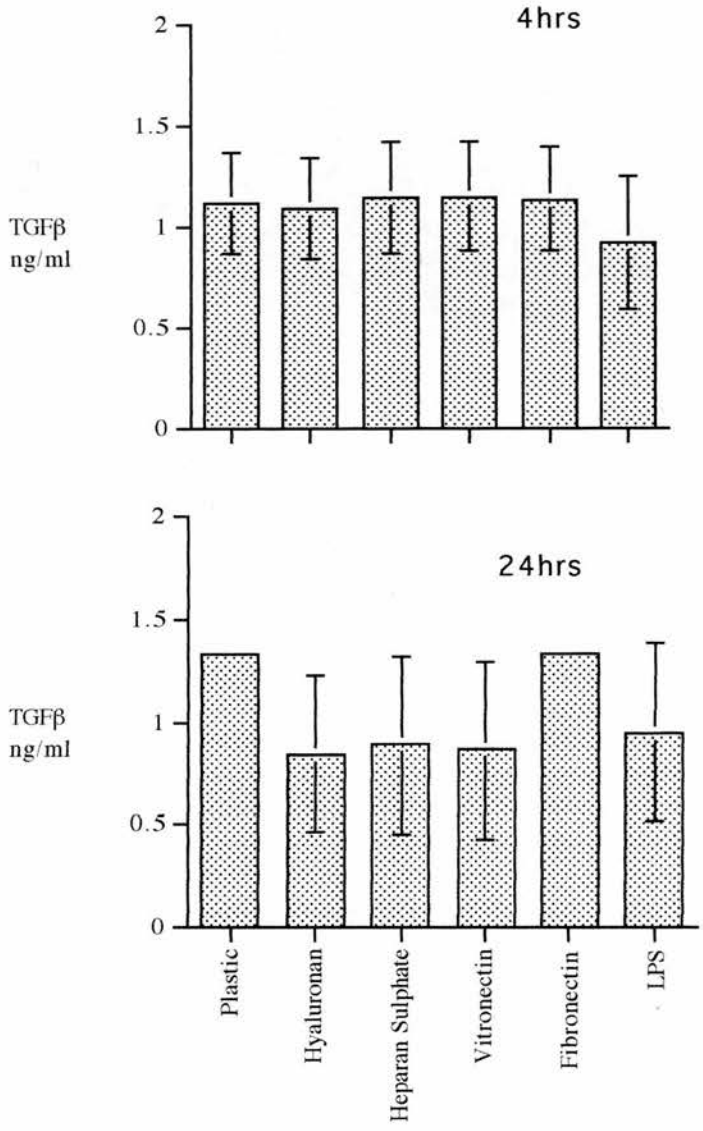


Figure 4.4

Effect of different ECM substrates on the release of TGFβ from adherence separated monocytes (n=4), TGFβ was assessed by ELISA. At the times investigated there were no significant differences in TGFβ release from monocytes on different substrates.

Neither IL-8 release (figure 4.3) nor TGFβ release (figure 4.4) were increased significantly by these ECM molecules or LPS at the given time points studied here.

4.2.2 LPS Neutralising Experiments

Since hyaluronan was not available as an endotoxin-free tissue culture grade reagent from any supplier, and the size of the molecule precluded use of the standard assay for the presence of LPS contamination (Limulus assay) a series of experiments were performed to eliminate the possibility that LPS contamination of hyaluronan preparations contributed to the observed effects. An antibody to LPS (WN1, kindly provided by Robin Barclay, S.E. Scotland Regional Blood Transfusion Centre, Royal Infirmary, Edinburgh.) was obtained which binds to several forms of LPS (Di Padova et al., 1993). Preliminary experiments pre-incubating WN1 at different concentrations with LPS (figure 4.5) prior to a 4 hour incubation with monocytes showed that WN1 did not inhibit the LPS-induced TNFα release in monocytes. Further experiments using monocyte-derived macrophages and WN1 also failed to inhibit LPS stimulation (data not shown). An alternative method was therefore pursued for assessing the contribution of LPS to TNFα release induced by hyaluronan.

Polymyxin-B (PXB) is an antibiotic that binds to the lipid A moiety of LPS, abrogating its activity (Morrison and Jacobs 1976). As PXB binds stochiometrically to LPS (3.5μg/ml PXB binds approximately 10μg/ml LPS) 4μg/ml was considered adequate. Pre-incubation of PXB at 4μg/ml and 0.4μg/ml with 1μg/ml LPS reduced TNFα release significantly (figure 4.6, $p<0.05$), but PXB did not affect hyaluronan induced TNFα release (n=11).

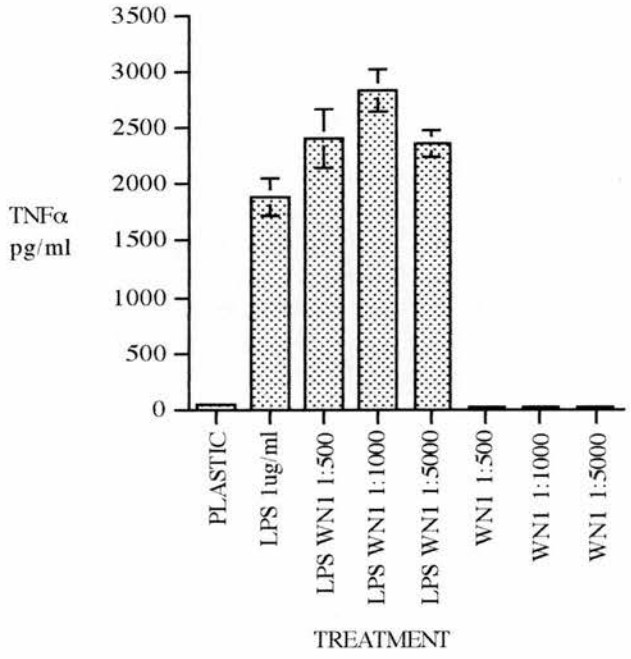


Figure 4.5 Pre-incubation of different concentrations of WN1 with LPS (1μg/ml) for 15 minutes did not inhibit the stimulatory effect of LPS on TNFα release (n =1, mean of triplicates ± standard deviation). Incubation of WN1 alone with monocytes had no effect on TNFα release.

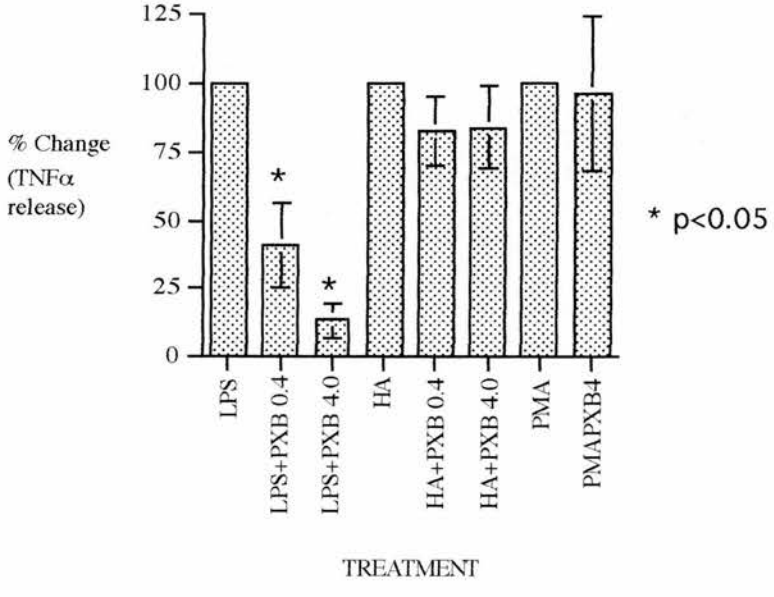


Figure 4.6 LPS (1μg/ml), tissue culture plates pre-coated with hyaluronan (HA) (1mg/ml), and PMA (1μM) were pre-treated with polymyxin B (PXB; 0.4μg/ml and 4.0μg/ml). Day 6 macrophages were then incubated for 4 hours under these conditions and TNFα was assessed by ELISA. PXB significantly reduced LPS-induced TNFα release ($p<0.05$) at both 0.4μg/ml (n=9, mean ± SEM) and 4.0μg/ml (n=7). PXB had no significant effect on HA- or on PMA-induced TNFα release.

PXB is also a known inhibitor of protein kinase-C (PKC), (Mazzei et al., 1982, Aida et al., 1990) which has a pivotal role in intracellular signal transduction pathways. Direct activation of PKC dependent pathways for TNFα production using phorbol myristate acetate (PMA) (Nishizuka 1984) could not be inhibited (figure 4.6), even at high concentrations of PXB. This suggests that PXB does not exert its effect by inhibition of PKC activity in this system. Together these data show that the TNFα release seen following adhesion of monocytes to hyaluronan is not due to trace contamination of hyaluronan preparations by LPS.

4.2.3 Quantitation of TNFα Release in Response to Hyaluronan

To assess whether TNFα production by adherent monocytes was influenced by hyaluronan in a concentration dependent manner, hyaluronan was pre-coated at different concentrations onto tissue culture plastic. Monocyte release of TNFα was found to increase in parallel with increased pre-coated concentration of hyaluronan (figure 4.7). I therefore determined the amount of hyaluronan bound to tissue culture plastic at different concentrations using biotinylated hyaluronan binding region (HaBR) which binds to the proteoglycan G1 domain (Fosang et al., 1990). Data shown in figure 4.8 indicate that hyaluronan concentrations above 1mg/ml did not increase optical density values, suggesting that hyaluronan binding may be maximal at 1mg/ml. However, given that monocyte TNFα release is augmented further at 4mg/ml pre-coated hyaluronan, it is possible that HaBR (1μg/ml) was limiting hyaluronan detection at 1mg/ml. Titration of HaBR against hyaluronan at 1mg/ml was found to be optimal at 1μg/ml (figure 4.9).

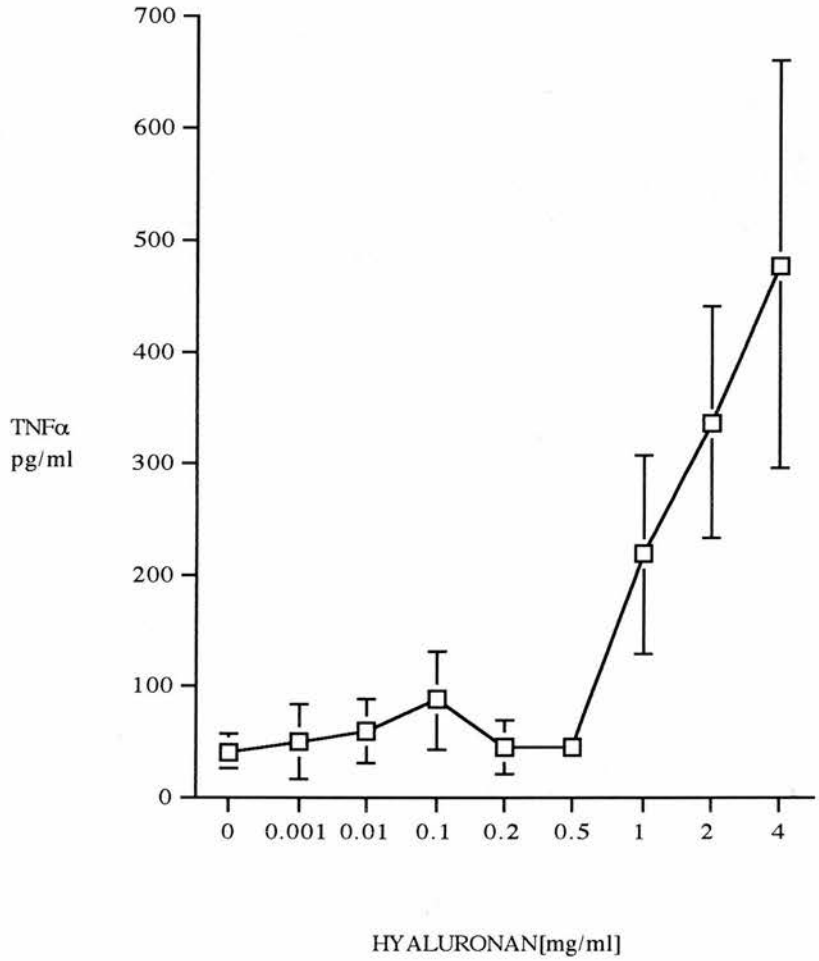


Figure 4.7

Monocytes were incubated for 4 hours on tissue culture plates pre-coated with increasing concentrations of hyaluronan (sonicated prior to use). Hyaluronan induced TNFα release in a dose dependent manner. Mean of 5 experiments ± standard error of the mean (SEM).

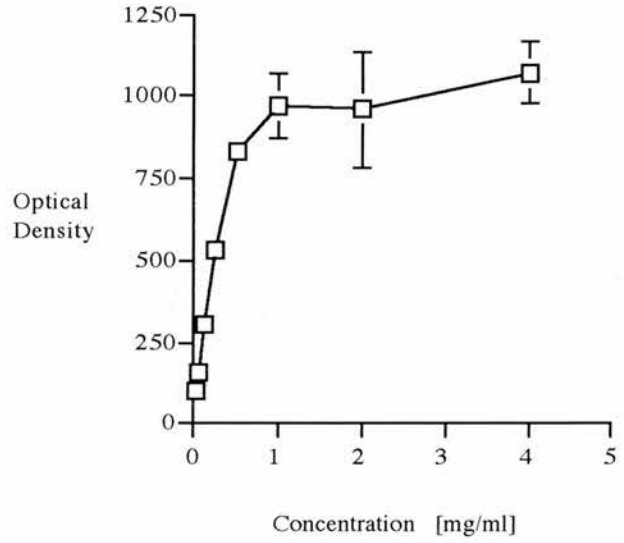


Figure 4.8

Assessment of hyaluronan bound to tissue culture plastic with hyaluronan binding region (biotinylated proteoglycan G1 domain; 1μg/ml). Hyaluronan binding appears to plateau at 1mg/ml on tissue culture plastic.

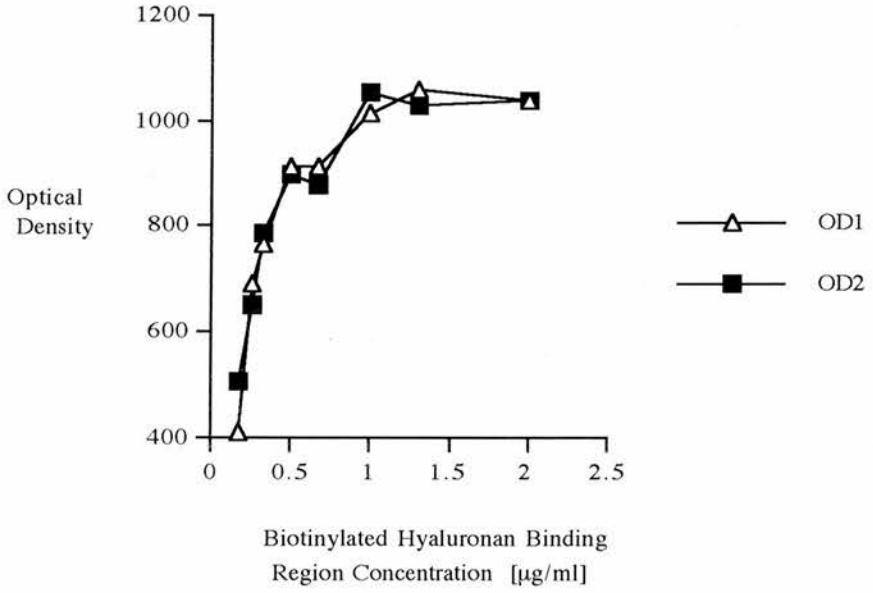


Figure 4.9

Biotinylated hyaluronan binding region (HaBR) was titrated against 1mg/ml hyaluronan tissue culture plastic. HaBR detection of hyaluronan appears to plateau at 1mg/ml. Results taken from one representative experiment OD1 and OD2 represent duplicate wells.

Compared to the other ECM molecules assessed in the initial cytokine release experiments, 1mg/ml hyaluronan may appear to be an excessively high concentration. However, the concentration used in this study is within the range used in other studies of the biological effects of hyaluronan. As all hyaluronan preparations were sonicated prior to use, the biological effects of hyaluronan in terms of molecular weight, in this study would also be supported by other studies.

Investigation of the effect of high ECM coating concentrations (figure 4.10, n=4) revealed that even when used at very high coating concentrations (5 times those used in most studies of the effects of ECM components) fibronectin and collagen still failed to induce significant TNFα release, supporting the suggestion that hyaluronan-induced effects are specific.

4.2.4 TNFα Protein Synthesis in Response to Hyaluronan

To further analyse the molecular mechanisms involved in hyaluronan induced TNFα release, the ability of monocytes to release TNFα when adhered to hyaluronan over 4 hours in the presence of the protein synthesis inhibitor cycloheximide was investigated. Pre-treatment of monocytes with 50mM cycloheximide for 30 minutes was sufficient to reduce hyaluronan-induced TNFα release by 75% reaching significance for both LPS- and hyaluronan-stimulated cells (figure 4.11, $p<0.01$, n=4). Consistent with a requirement for new protein synthesis in the release of TNFα by hyaluronan-adherent monocytes, TNFα mRNA was detected by RT-PCR. Detailed temporal analysis revealed that TNFα mRNA was transiently expressed after 2 hours of monocyte adhesion to hyaluronan, (figure 4.12a), PCR for GAPDH

indicated that all the samples contained RNA (figure 4.12b). In contrast, TNFα protein release was barely detectable at 2 hours (at the lower detection limit of the ELISA) but increased significantly at 4 hours. Together these data indicate that in monocytes, hyaluronan induces TNFα transcription leading to new protein synthesis and subsequent release.

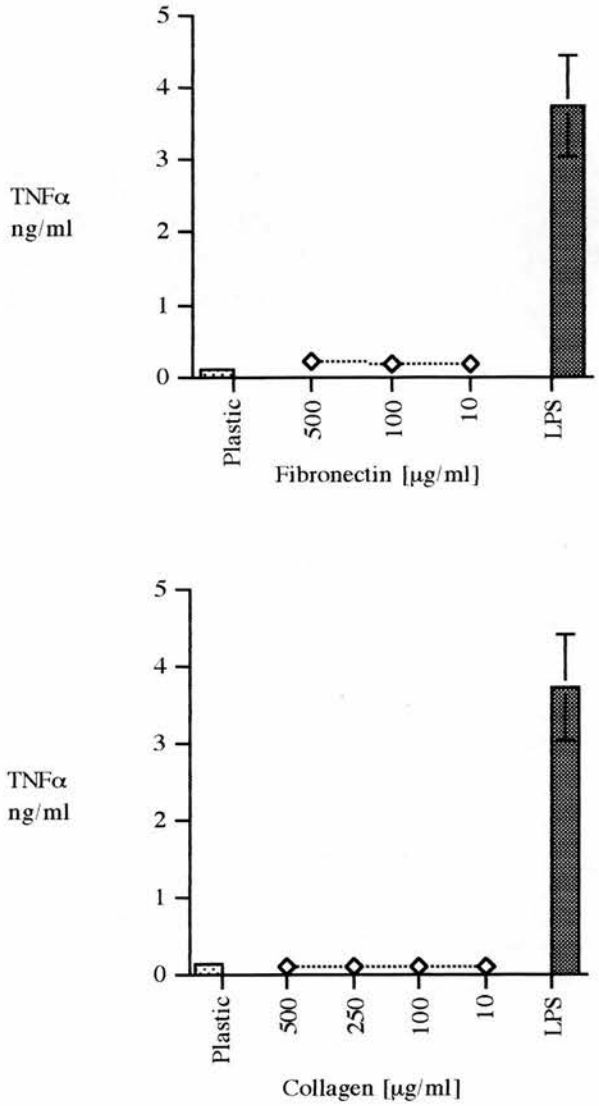


Figure 4.10

Monocytes were incubated for 4 hours on tissue culture plastic pre-coated with increasing concentrations of fibronectin and collagen. No significant increase in TNFα release was seen at these concentrations.

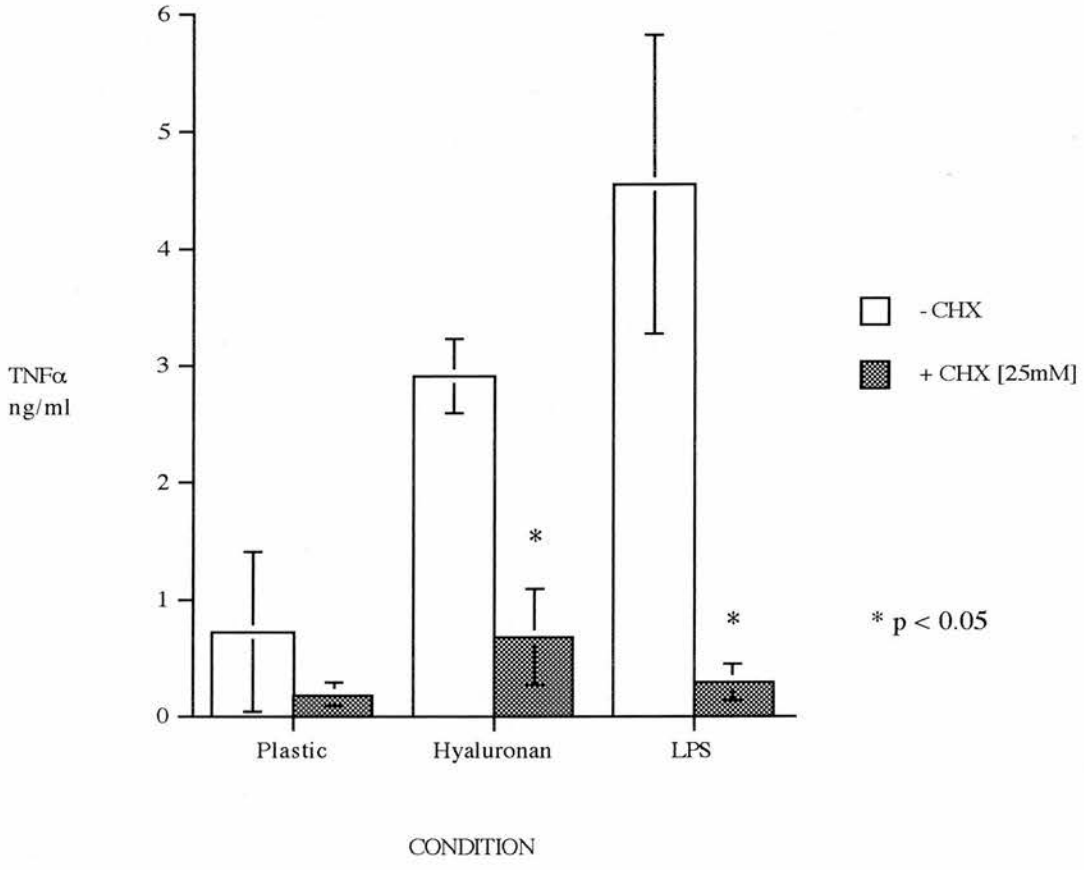


Figure 4.11

Monocytes were either pre-treated with 50μM cycloheximide (CHX), or pre-incubated in medium alone, for 30 minutes. Cells were then incubated for 4 hours on plastic, hyaluronan-coated plastic (1mg/ml), or with LPS (1μg/ml). Cycloheximide in pre-treated cells was diluted to a final concentration of 25μM for the 4 hour incubation period. Both hyaluronan- and LPS-induced TNFα release were decreased significantly ($p < 0.05$) with cycloheximide.

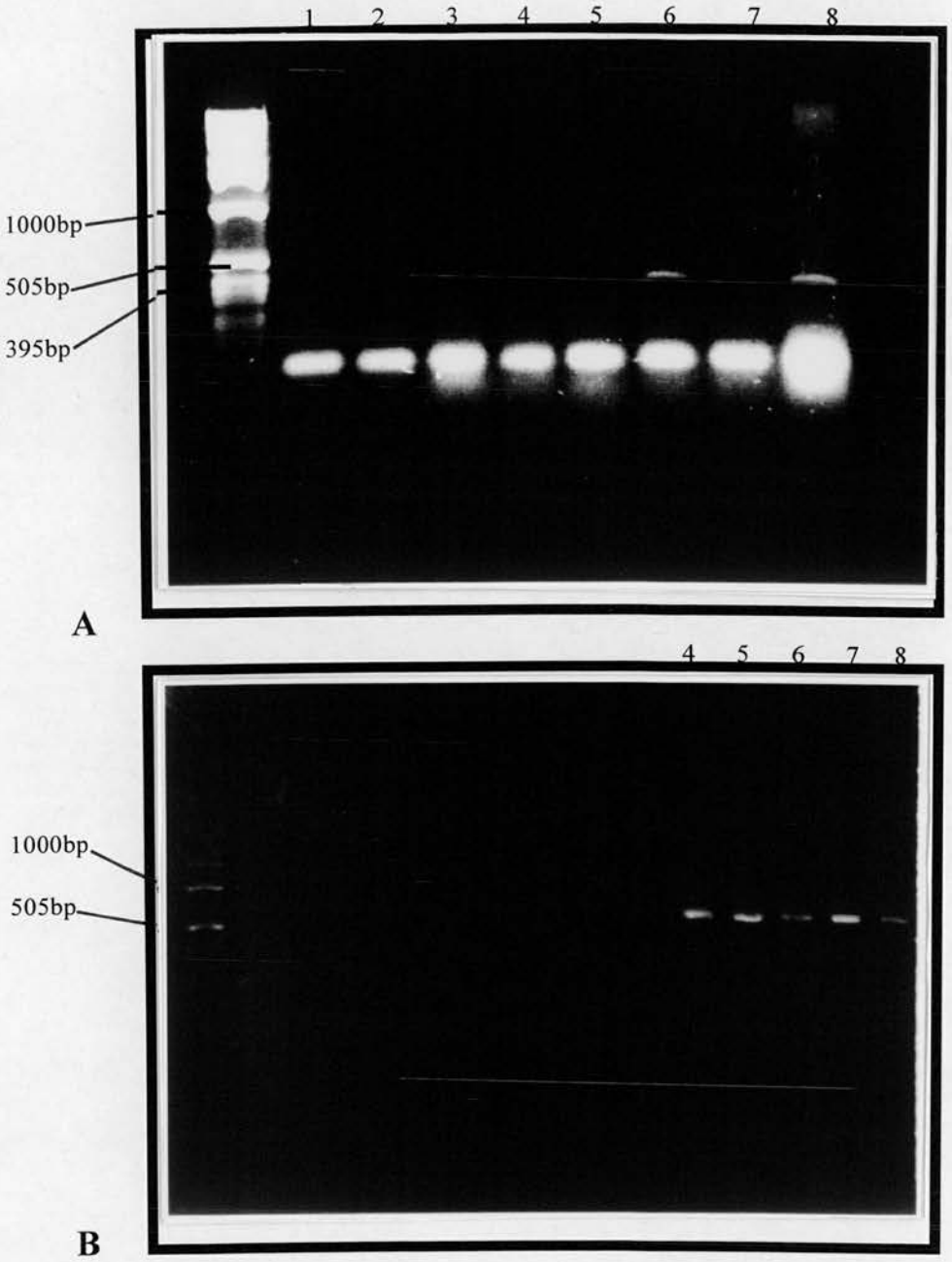


Figure 4.12

A. Monocytes were allowed to adhere to tissue culture plastic pre-coated with hyaluronan for 30 minutes (lane 4), 1 hour (lane 5), 2 hours (lane 6), and 4 hours (lane 7). RNA was extracted and amplified for TNFα mRNA by RT-PCR. Lane 8 shows the positive control (phytohemagglutinin-stimulated peripheral blood lymphocytes). Lanes 1-3 represent negative controls. TNFα message is clearly visible at 2 hours (lane 6)

B. Positive controls (GAPDH) for the same samples were run on another gel, visible bands correspond to lanes 4-8 in (A).

Both gels were run with 1kb DNA ladders for reference.

4.3 Discussion

Several studies have indicated that monocytes incubated with LPS produce cytokines (Beutler and Cerami 1986, Morrison and Ryan 1987). LPS was used in this system as a positive control. Data presented in this thesis confirm monocyte release of TNFα and IL-1β (Matthews 1981, Mayernik 1984) in response to LPS. However, neither IL-8 nor TGFβ release appeared to be affected by LPS treatment of adherence separated monocytes.

IL-8 is constitutively released by adherent monocytes (Kasahara et al., 1991), published levels have ranged from 10ng/ml (Dentener et al., 1993, 1993a), to 32ng/ml (Hallsworth et al., 1994). IL-8 release in monocytes has also previously been shown to be enhanced in response to LPS (Peveri et al., 1988) and reported levels range from 100ng/ml (Heinel et al., 1995) to 400ng/ml (Dentener et al., 1993). These differences in IL-8 release upon stimulation with LPS may reflect different cell isolation procedures; the studies showing a significant release with LPS used monocytes isolated from buffy coat preparations. It is possible that the buffy coat monocytes were primed prior to stimulation with LPS whereas those studies where no significant increase in release was seen (including my own) used freshly isolated monocytes from peripheral blood. Alternatively, discrepancies in reported levels may be explained by a recent WHO collaborative study (Mire-Sluis et al., 1997) which indicates much inter-laboratory variation of IL-8 assays, which could result in a 5-10 fold difference in IL-8 estimates for the same sample in different laboratories. So although absolute values for IL-8 release may be questionable, the relative release is of course a useful indicator of the cell activation state.

LPS also failed to increase TGFβ release in my experiments. Although TGFβ mRNA was found to be constitutively expressed in monocytes and macrophages (Assoian et al., 1987) and monocytes isolated from the synovial fluid of patient with rheumatoid arthritis secreted active TGFβ, peripheral blood monocytes released only small amounts (1.4ng/ml) of latent TGFβ (Wahl et al., 1990). This level is comparable to the constitutive TGFβ release seen in my experiments. However, both Assoian et al., (1987) and Wahl et al., (1990) found that LPS (at 10μg/ml and 1μg/ml respectively) increased TGFβ release over 24-48 hours. It is likely that 1μg/ml over the shorter time periods in my experiments (4 and 24 hours) was not sufficient to significantly enhance TGFβ release.

Monocyte adherence to fibronectin, vitronectin, and heparan sulphate did not result in any significant cytokine release in this system. Fibronectin has previously been shown to stimulate monocytes and macrophages to release TNFα (Beezhold and Personius 1992, Peat et al., 1995), and IL-1β (Graves and Roman 1996), however these studies investigated the effects of exogenous fibronectin added in solution to cells adherent to plastic. Although adherence to plastic alone may be sufficient to induce IL-8 release (Kasahara et al., 1991) and to induce the expression of mRNA for CSF-1 and TNFα (Haskill et al., 1988, Eierman et al., 1989), Haskill et al., (1988) found that a second stimulus (LPS) was necessary for the translation and secretion of TNFα and CSF-1. Previously vitronectin has been shown to enhance TNFα production in macrophages when bound to beta-glucan (Olson et al., 1996), i.e., when acting in concert with another stimulus. It is possible that in studies in which

soluble fibronectin triggers TNFα production, the monocytes are primed by the adhesion to plastic and the fibronectin is acting as a second stimulus. This suggestion could also explain the lack of cytokine induction in response to monocyte adherence to vitronectin, seen in my studies.

In contrast to the other ECM substrates investigated, notably the structurally related glycosaminoglycan heparan sulphate, adherence to hyaluronan resulted in a release of TNFα, which was dose dependent. Over longer times (24 hours) the TNFα release decreased, this may have been due to a toxic effect of hyaluronan on monocytes (discussed in chapter 5). Monocyte sequestration and internalisation of TNFα has not been reported, but remains as a possible explanation for reduced levels of TNFα production at longer time points. Alternatively hyaluronan may have been degraded by monocytes, thereby neutralising its pro-inflammatory effect. Although macrophages have been implicated in the uptake and degradation of hyaluronan (Underhill et al., 1993), it is unlikely that this occurs in monocytes (see chapter 5). However, this possibility could be addressed by assessing the hyaluronan present on the tissue culture plastic after removing the monocyte monolayer. Longer time points may also be complicated by monocyte production and deposition of matrix proteins. The extent to which this occurs could be ascertained through the use of ECM-binding proteins, or antibodies, after the removal of the monocyte monolayer. These complications may be minimised by considering data from shorter time points. Although hyaluronan-induced TNFα release increased with hyaluronan concentration, hyaluronan binding to tissue culture plastic was found to plateau at 1mg/ml, detected with hyaluronan binding region (HaBR). It is possible that

assessment of binding was limited by the HaBR concentration, but considering the detection limits of such a colorimetric assay it is unlikely that increased concentration of HaBR would generate a higher optical density. At higher concentrations the viscosity of hyaluronan may influence immunomodulatory actions. At concentrations of above 1mg/ml, molecular entanglement occurs in hyaluronan solutions (Ogsten and Stanier 1953) which tend to form a continuous network and therefore affect physical interactions with cells, below 1mg/ml hyaluronan molecules remain separate (Laurent et al., 1960).

Adhesion to hyaluronan was sufficient to induce both TNFα mRNA at 2 hours, and significant secreted TNFα and IL-1β protein at 4 hours. Although TNFα mRNA is also transiently induced by LPS, and has been found to peak at 2 hours post-stimulation (Kohn 1992, Donnelly et al., 1995), LPS contamination was excluded in my system by experiments in which the effects of polymyxin-B were assessed. Polymyxin-B inactivates LPS activity (Morrison and Jacobs 1976) and did not affect the hyaluronan-induced TNFα release.

Polymyxin-B has also been shown to inhibit PKC activity (Mazzei et al., 1982, Aida et al., 1990), which is one pathway by which LPS activation can occur (reviewed by Sweet and Hume 1996). In fact experiments using PMA, which directly activates monocytes through a PKC-dependent pathway, indicated that the concentration of polymyxin-B was not sufficient to inhibit PMA induced PKC activation. Thus it is likely that the marked reduction in LPS-induced TNFα release was due to the specific interaction between polymyxin-B and the lipid A portion of LPS, and not through the inhibition of PKC activity.

TNFα mRNA expression has been reported in murine macrophages in response to soluble hyaluronan (Noble et al., 1993). Expression of TNFα mRNA was induced at 1 hour following stimulation with 1mg/ml soluble hyaluronan and was sustained for 12 hours. The difference in mRNA expression over time in human monocytes may reflect species differences, although this difference was not investigated. TNFα release from human monocytes was also induced in response to soluble hyaluronan (see chapter 5). It is interesting that the kinetics of hyaluronan-induced TNFα mRNA expression differs from that of adherence to plastic which has been reported to induce TNFα mRNA transiently after as little as 20 minutes (Haskill et al., 1988). In contrast Eierman et al. (1989) found sustained expression from 40 minutes over 12 hours. However, Eierman et al., used monocytes from platelet pheresis bags (buffy coats) which again may have influenced the cell activation state.

The hyaluronan-induced production and release of TNFα and IL-1 in human monocytes is supported by previous studies in other species. Soluble hyaluronan has previously been shown to stimulate IL-1-like activity in rabbit peritoneal macrophages and human monocytes (Hiro et al., 1986). More recently, hyaluronan induced mRNA transcripts for TNFα and IL-1β, and increased constitutive IGF-1 synthesis in murine bone marrow-derived macrophages (Noble et al., 1993) which was shown to be dependent on TNFα expression. Interestingly, hyaluronan has also been shown to increase IL-8 mRNA in alveolar macrophages from patients with idiopathic pulmonary fibrosis (McKee et al., 1996). Although IL-8 mRNA was not assessed in the present studies, it is possible that, like the TNFα induced IGF-1 release, TNFα may also affect the production of IL-8. TNFα has previously been

shown to induce IL-8 expression in human astrocytoma cell lines (reviewed in Chouaib et al., 1991). To address this question TNFα neutralising antibodies could be incorporated to block any TNFα induced activity. This would be an interesting experiment to carry out, as it would provide more insight to the network of cytokines involved in the monocyte/macrophage response to hyaluronan.

Hyaluronan-induced release of TNFα was significantly reduced in the presence of cycloheximide, in conjunction with this TNFα mRNA data, significant release of TNFα protein was not detected before 4 hours, indicating that de novo protein synthesis is required. These data do not preclude cleavage from membrane bound protein, or intracellular granules as contributory mechanisms for release. To assess these possibilities fluorescent labelled anti-TNFα antibodies, or intra-cellular staining of fresh monocytes could be employed, although low levels of TNFα would be difficult to detect. Alternatively, inhibitors of TACE could be incorporated which would prevent cleavage of membrane-bound TNFα.

Recent studies indicate the molecular weight of hyaluronan is an important influence over cell responses. McKee et al., (1996) found that low molecular weight fragments of hyaluronan (less than $5x10^5$D) were stimulatory for chemokine gene expression whereas higher molecular weight forms failed to induce gene expression. Although Hiro et al., (1986) found that low molecular weight hyaluronan from rooster comb induced less IL-1 from monocytes, the higher weight form, from human umbilical cord, was estimated at $8x10^5$D, which is smaller than the inactive preparations used by McKee et al., which were in excess of 10^6D. The results in this thesis would

support the pro-inflammatory effects of low molecular weight hyaluronan as all hyaluronan preparations were sonicated prior to use.

The results presented in this chapter indicate that hyaluronan, in contrast to fibronectin, vitronectin, collagen, and heparan sulphate, induces monocyte production and release of TNFα, and to a lesser extent, the release of IL-1β. These pro-inflammatory effects of hyaluronan are examined further in chapter 5.

Chapter 5

5.1 Introduction

5.1.1 Biological Importance of Hyaluronan

The physiological importance of hyaluronan is highlighted by its presence from the beginning of embryonic development. Hyaluronan is necessary for the migration of mesenchymal cells into the blastocoel (Fisher and Salrush 1977), and later in development of the neural crest (reviewed in Erickson and Perris 1993).

High levels of hyaluronan are seen in normal developing murine lungs (Underhill et al., 1993). As development progresses the ratio of hyaluronan to protein content decreases and little hyaluronan has been observed in normal adult rat (Nettlebladt 1989) or mouse models (Underhill et al., 1993).

In compensatory lung growth after resection of rat lungs (Mueller and Thet 1987) high levels of hyaluronan are also observed, thus supporting a role for hyaluronan in tissue remodelling.

5.1.2. Hyaluronan and CD44 in Wound Healing

Wound healing shares several features in common with embryonic development, particularly with respect to ECM turnover (Chapter 1, section 1.9). Hyaluronan is deposited early in the wound healing process (Bentley 1967, 1968) is a component of the provisional matrix (Weigel et al., 1986) and has been shown to accelerate fibrin clot formation (LeBoeuf et al., 1986). Many of the biological effects of hyaluronan are mediated through the cell surface receptor CD44. Fibroblast migration on

hyaluronan is enhanced by CD44 expression (Peck and Isacke 1996) and fibroblast migration into a fibrin matrix is inhibited by antibodies to CD44 (Svee et al., 1996). In view of the observation that hyaluronan induces the production of pro-inflammatory cytokines from monocytes (Chapter 4), its accumulation after injury may sustain, or enhance the inflammatory response. One of the central hypotheses of this thesis is that where inflammatory and remodelling processes are not held in equilibrium, disease pathogenesis may occur. Accumulation of hyaluronan after injury in adults may paradoxically enhance the inflammatory response, as adhesion to hyaluronan induces production of pro-inflammatory cytokines from monocytes. The pulmonary fibrotic disorders sarcoidosis and idiopathic pulmonary fibrosis, are two examples with unknown aetiology where increases in hyaluronan have previously been found (Hallgren et al., 1985, Bjermer 1989) and may reflect this imbalance.
In this chapter, the presence of hyaluronan in lung tissue and bronchoalveolar lavage fluid was investigated and the influence of hyaluronan on inflammatory responses was examined further.

5.2 Results

5.2.1 Quantitation of Hyaluronan in BAL

The levels of hyaluronan in samples of BAL fluid from patients at risk of ARDS and with established ARDS was assessed by competitive ELISA (Fosang et al., 1990), and compared with levels found in healthy volunteers (smokers, n=28 and non smokers, n=8)(figure 5.1). No significant differences in levels of hyaluronan were noted between smokers and non-smokers from healthy volunteers, thus these values were taken together as normal controls. Levels of hyaluronan varied widely in each group; in established ARDS lavage fluid, hyaluronan levels ranged from 8ng/ml to 800ng/ml with a mean value of 179.0 ± 56.8, whereas in the at risk group hyaluronan levels ranged from 0 to 122ng/ml (mean; 21.4 ± 6.1), with a similar range for normal controls; 0 to 77ng/ml (mean; 22.8 ± 2.8).

When compared using non-parametric statistical analyses (Mann Whitney-U test), hyaluronan in the ARDS group was significantly higher than the corresponding levels in the normal control group (p=0.009). Although the numbers of at-risk patients who progressed to ARDS were low (n=4), there was a trend towards intermediate values between healthy controls and established ARDS. It is possible that increases in hyaluronan in BAL fluid could occur early in lung damage in patients who progress to ARDS and thus be indicative of the disease progression, however further BAL sample analyses would be required to establish this.

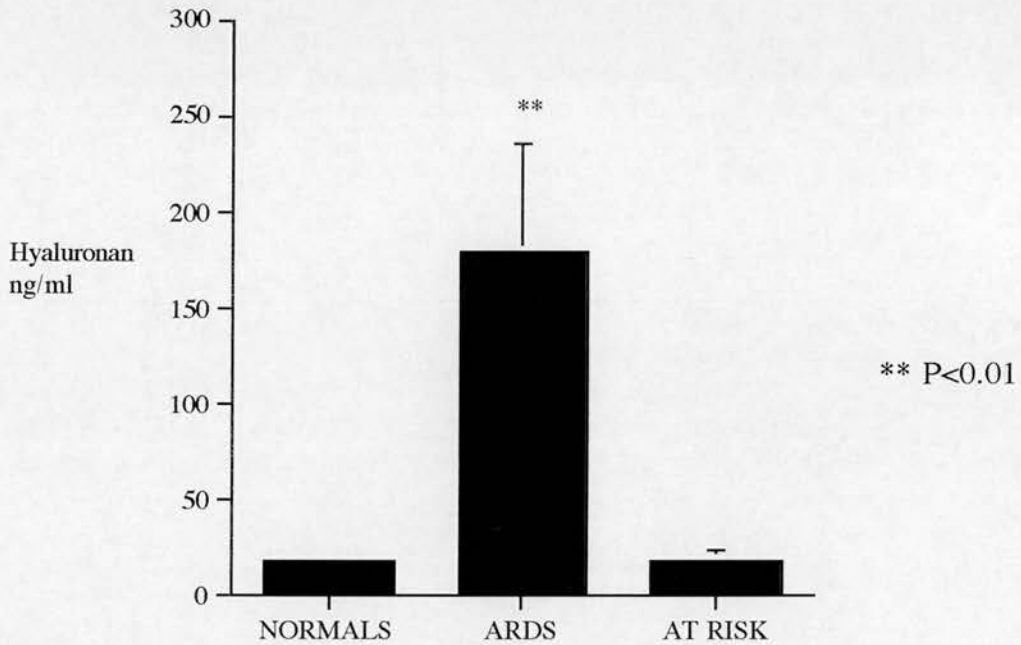


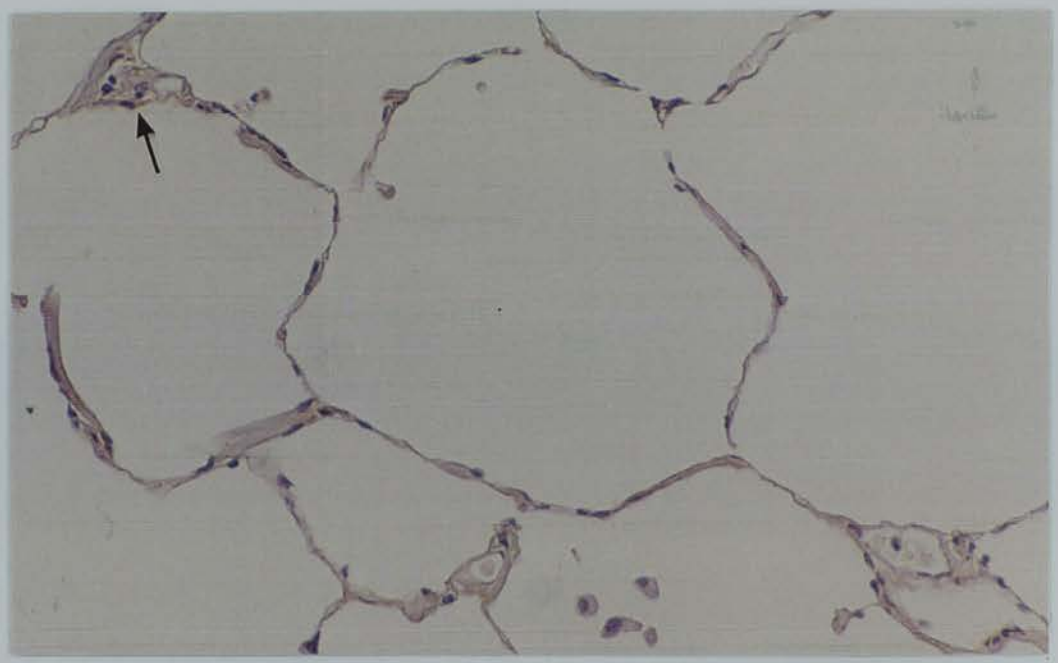
Figure 5.1

Bronchoalveolar lavage fluid from patients with ARDS (n=17), at risk of developing ARDS (n=27), and normal volunteers (n=36) was analysed for hyaluronan by competitive ELISA. Significantly elevated levels of hyaluronan were found in samples taken from patients with ARDS (p=0.009) compared with normal controls. There was no significant difference between patients at risk of ARDS and normal controls.

5.2.2 Localisation of Hyaluronan in Lung Tissue Sections

To further investigate the relationship between hyaluronan and disease pathogenesis in the lung, immunohistochemical staining (figure 5.2) of lung sections was undertaken. This study revealed the extent of hyaluronan present within the alveolar spaces and interstitium in lung tissue from patients who died from ARDS. This analysis was extended to localisation of hyaluronan in lung tissue from patients with sarcoidosis, cryptogenic fibrosing alveolitis, and tissue without evidence of interstitial lung disease (figure 5.3). The extent of staining in these groups is less than that in the ARDS tissue, suggesting that hyaluronan is less prominent in these disease states. Negative controls included slides stained with HaBR that had been pre-adsorbed to hyaluronan in solid phase (figure 5.3.3).

A



B

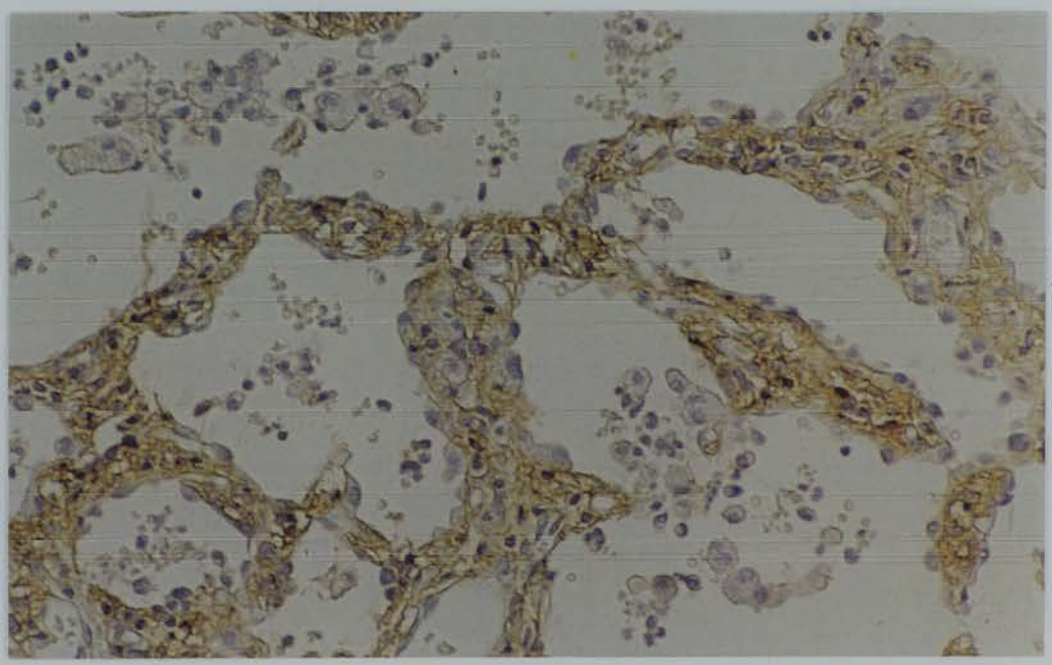
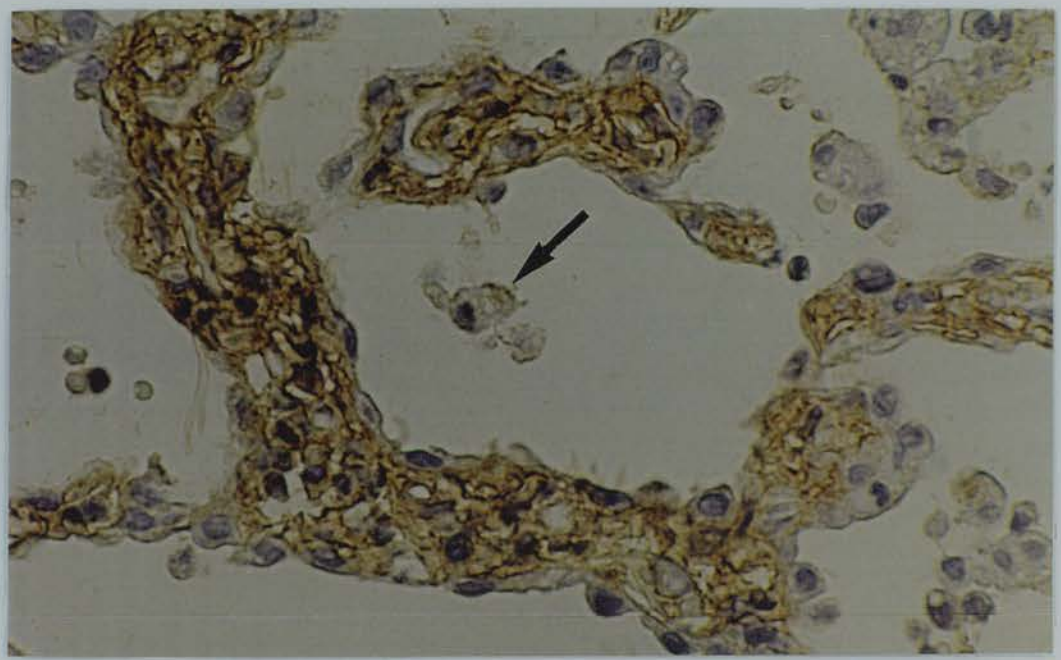


Figure 5.2.1
Lung tissue sections were stained for hyaluronan by indirect immunohistochemistry.
A. Photomicrograph of a normal lung tissue section without evidence of interstitial lung disease, slight staining for hyaluronan is visible around blood vessels (arrows).
B. Photomicrograph of a lung tissue section taken from a patient who died from ARDS showing thickened alveolar walls and extensive staining for hyaluronan particularly within the interstitium. (Both photographed using a x 20 objective)

A



B

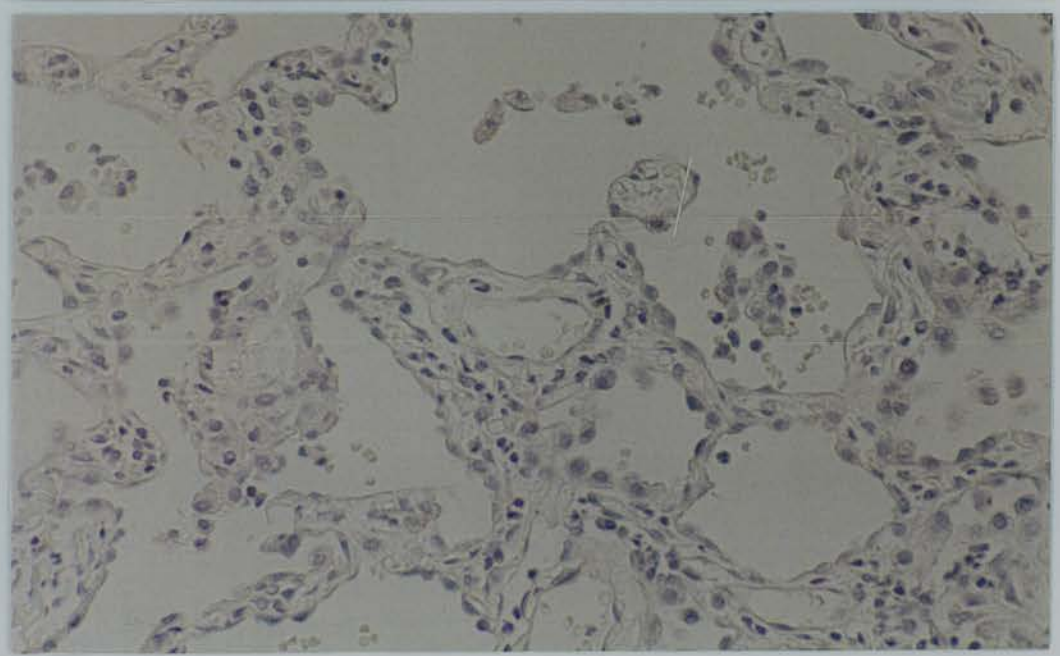
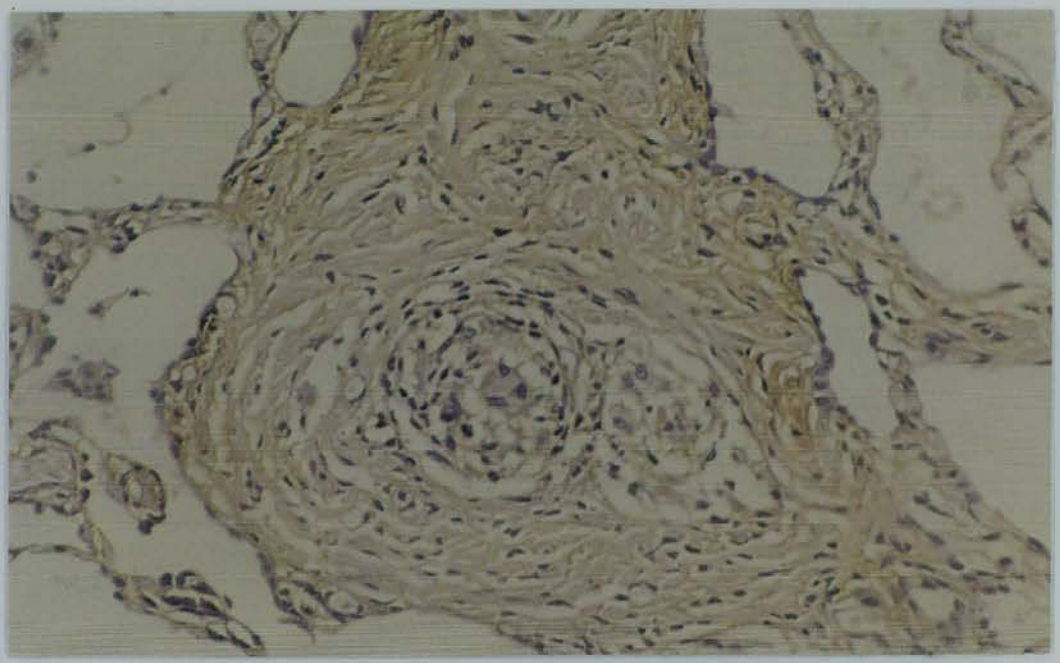


Figure 5.2.2
Lung tissue sections were stained for hyaluronan by indirect immunohistochemistry.

A. Photomicrograph of a lung tissue section taken from a patient who died from ARDS showing extensive staining for hyaluronan in the interstitium, and slight staining is apparent within an alveolar macrophage (arrow). (Photographed using a x 40 objective).

B. Photomicrograph of ARDS tissue negative control. (Photographed using a x 20 objective).

A



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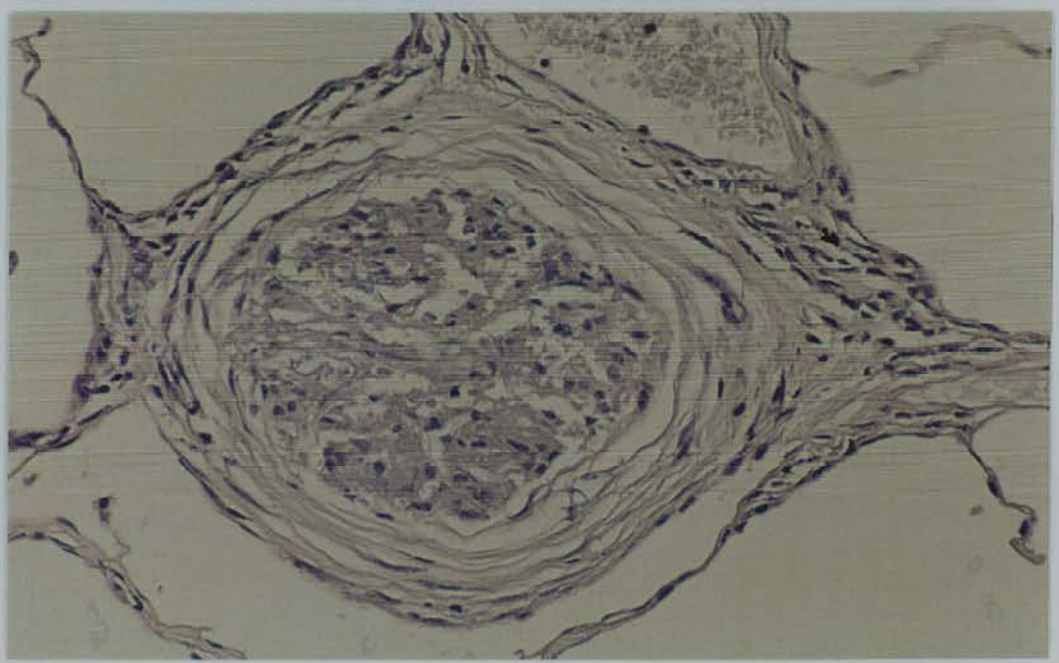


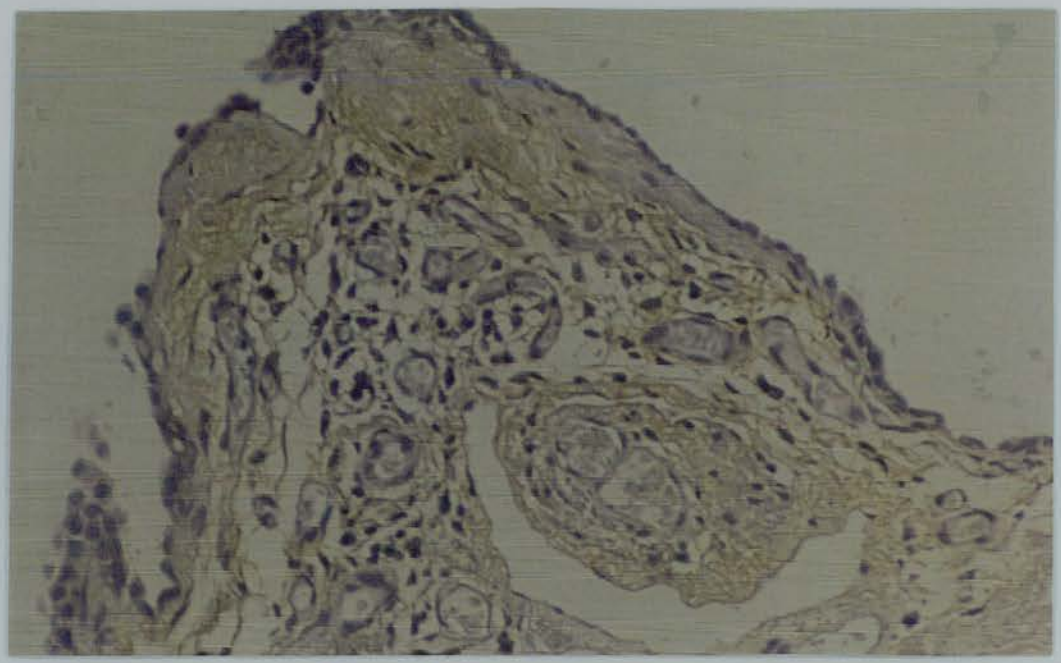
Figure 5.3.1
Lung tissue sections were stained for hyaluronan by indirect immunohistochemistry.

A. Photomicrograph of lung tissue section taken from a patient with sarcoidosis showing slight staining for hyaluronan particularly around peripheral granuloma tissue.

B. Photomicrograph of sarcoidosis tissue negative control.

(Both photographed using a x 20 objective)

A



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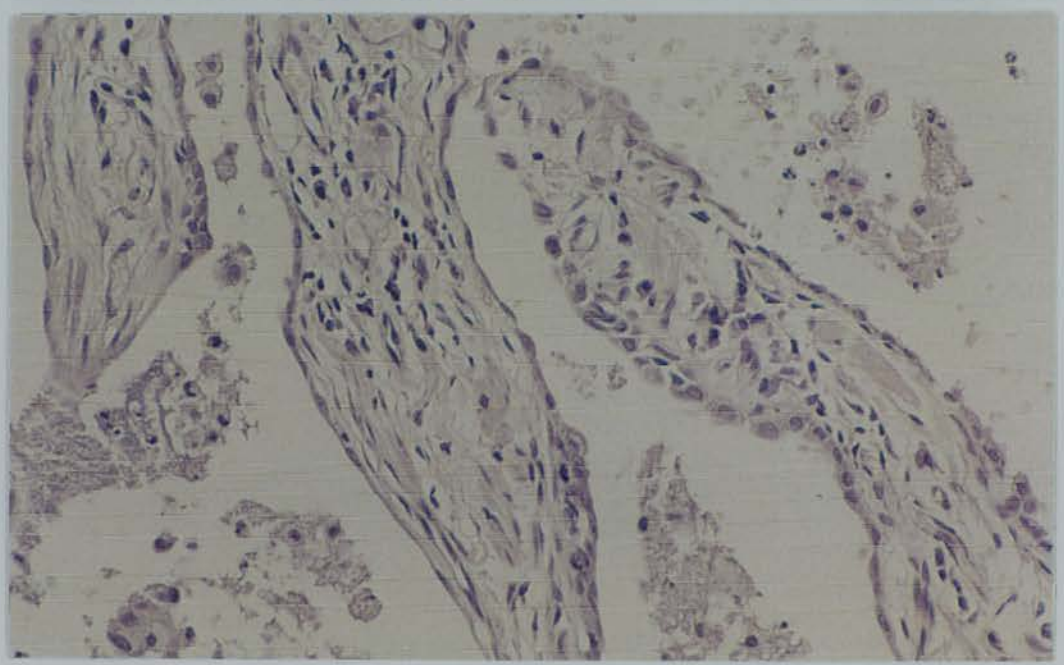


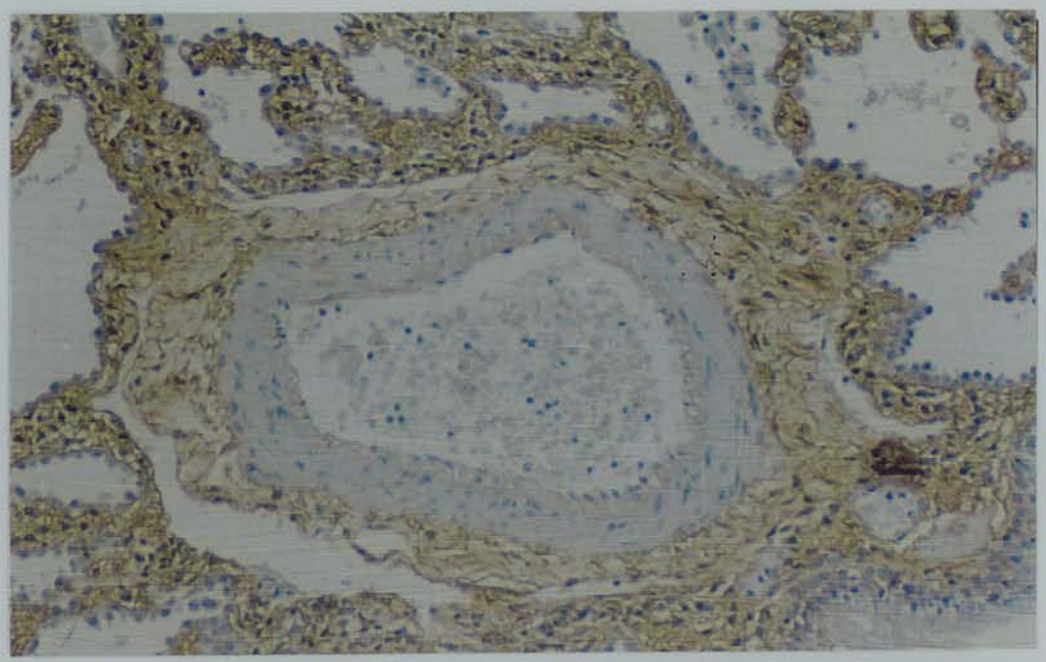
Figure 5.3.2
Lung tissue sections were stained for hyaluronan by indirect immunohistochemistry.

A. Photomicrograph of lung tissue section taken from a patient with cryptogenic fibrosing alveolitis (or idiopathic pulmonary fibrosis) showing slight staining for hyaluronan within the matrix of fibrotic alveolar walls.

B. Photomicrograph of cryptogenic fibrosing alveolitis tissue negative control.

(Both photographed using a x 20 objective)

A



B

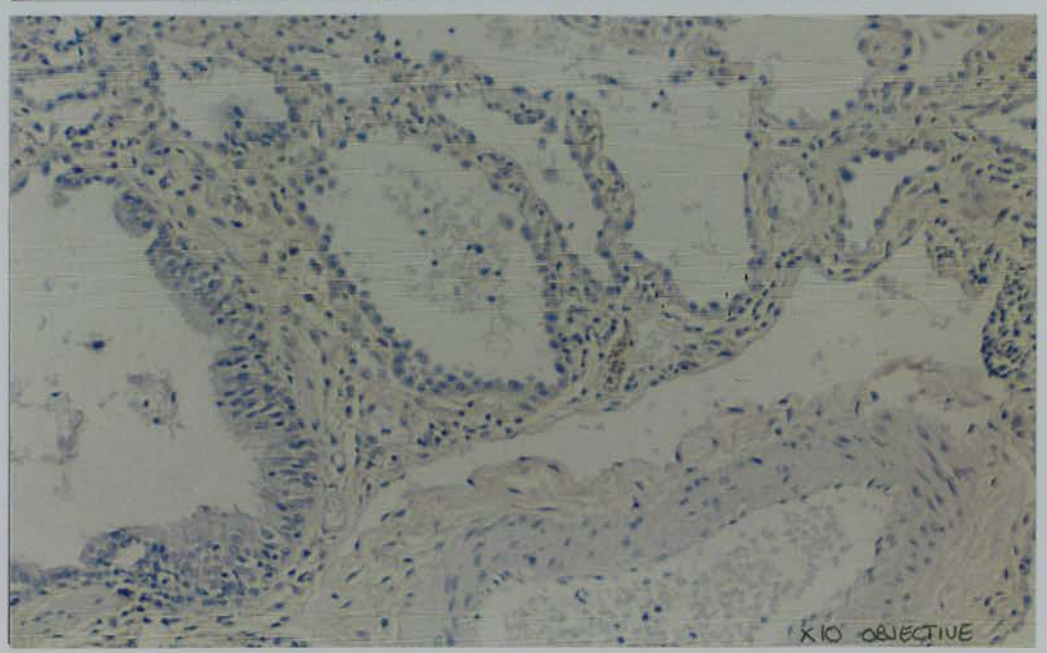


Figure 5.3.3

A. Photomicrograph showing tissue taken from a patient with ARDS, stained for hyaluronan with HaBR. As previously, extensive staining can be seen.

B. Photomicrograph showing tissue stained with the same concentration of HaBR previously adsorbed to hyaluronan in solid phase. This resulted in a marked loss of staining thus controlling for the possibility of non-specific staining.

5.2.3 Expression of CD44 in monocytes

As a complementary approach to examining lung tissue in ARDS samples for hyaluronan, the expression of the principal receptor for hyaluronan, CD44, was investigated. In particular the expression of variant isoforms of CD44, which have been associated with cellular migratory potential and the ability to bind to hyaluronan, were investigated during *in vitro* culture of monocytes.

The expression of CD44 in monocytes was rapidly up-regulated during *in vitro* culture (figure 5.4), especially the variant isoforms v3 and v10 (figure 5.5). There were no significant differences in CD44 expression in cells cultured on hyaluronan-coated plastic or cells on plastic alone. The differential expression of CD44 isoforms on monocytes and macrophages raised the possibility that the response of culture-derived macrophages to hyaluronan may differ from that of monocytes (figure 5.6).

5.2.4 Involvement of CD44 in Hyaluronan -induced TNFα release

Both monocytes and macrophages produce TNFα in response to hyaluronan, although use of the CD44 monoclonal antibody 5A4 revealed possible differences in the underlying regulatory mechanisms.

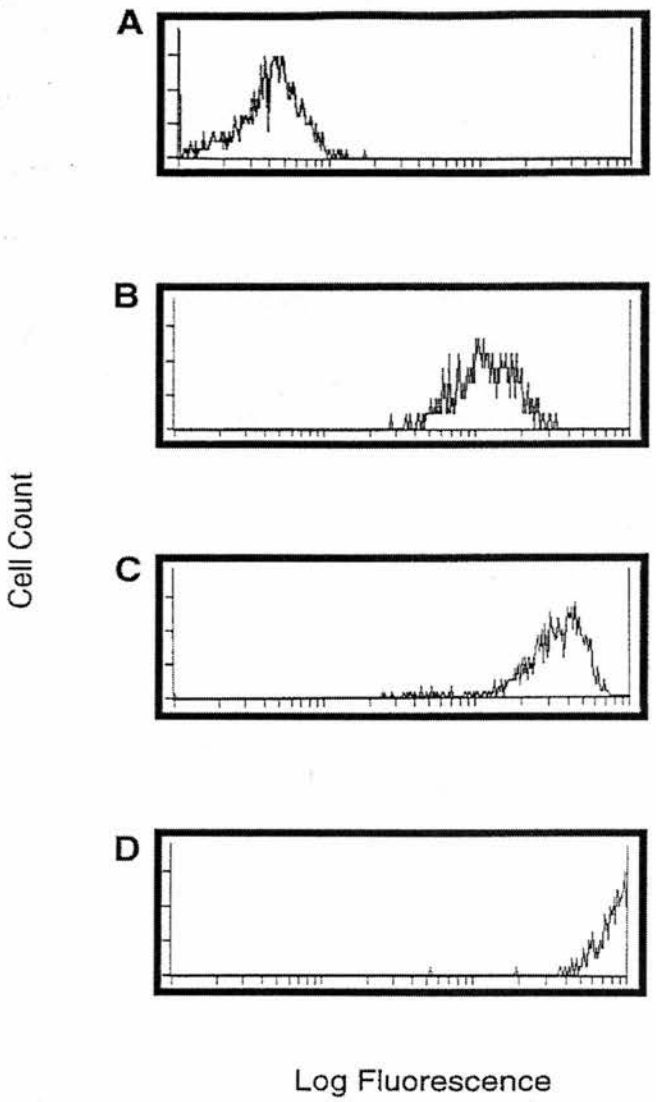


Figure 5.4.1

Monocyte expression of CD44 was assessed by indirect immunoflourescence and flow cytometry, using the monoclonal antibody 5A4, which binds all forms of CD44. Histograms are from one representative experiment of 4 that were performed.

A: Negative control, B: Freshly isolated monocytes, C: monocytes adherent to plastic for 4 hours, D: monocytes adherent to plastic for 24 hours.

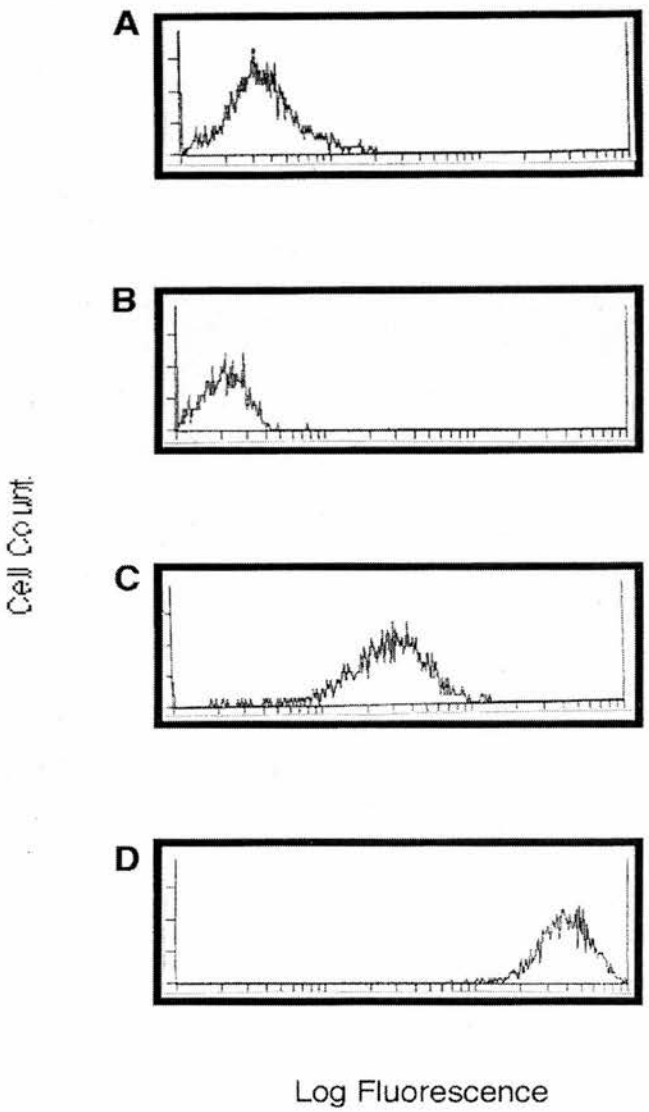


Figure 5.4.2

Monocyte expression of CD44 was assessed by indirect immunofluorescence and flow cytometry, using the monoclonal antibody 7F4, which defines an epitope associated with hyaluronan-binding (G. Dougherty, personal communication). Histograms are from one representative experiment of 4 that were performed.

A: Negative control, B: Freshly isolated monocytes, C: monocytes adherent to plastic for 4 hours, D: monocytes adherent to plastic for 24 hours.

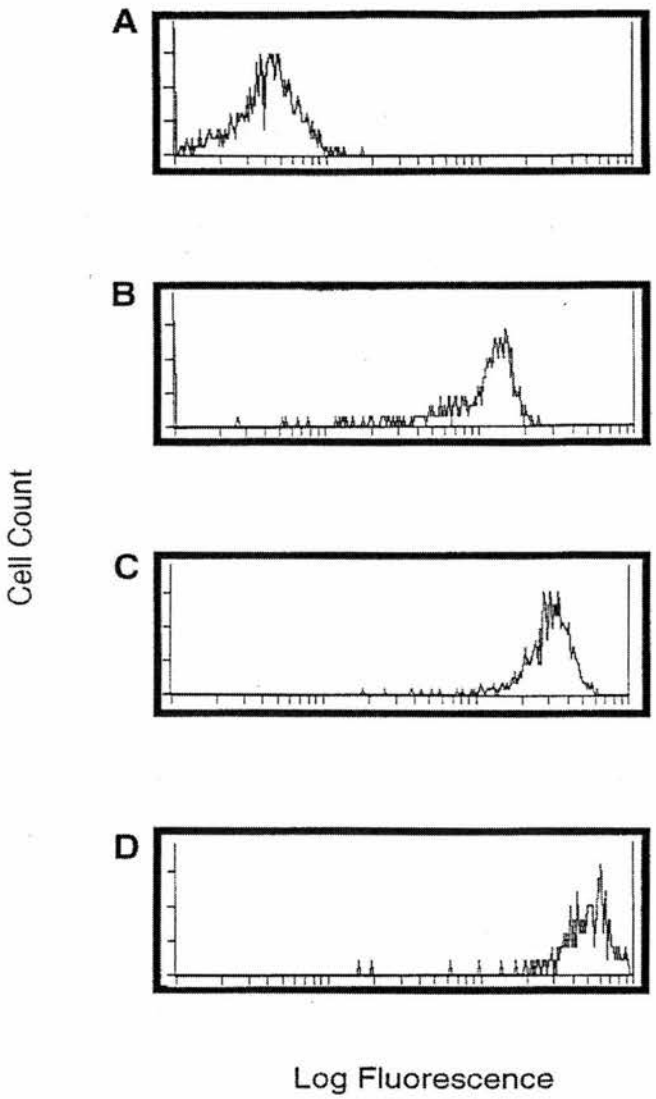


Figure 5.4.3

Monocyte expression of CD44 was assessed by indirect immunofluorescence and flow cytometry, using the monoclonal antibody 5A4, which binds all forms of CD44. Histograms are from one representative experiment of 4 that were performed.

A: Negative control, B: Freshly isolated monocytes, C: monocytes adherent to hyaluronan-coated plastic for 4 hours, D: monocytes adherent to hyaluronan-coated plastic for 24 hours.

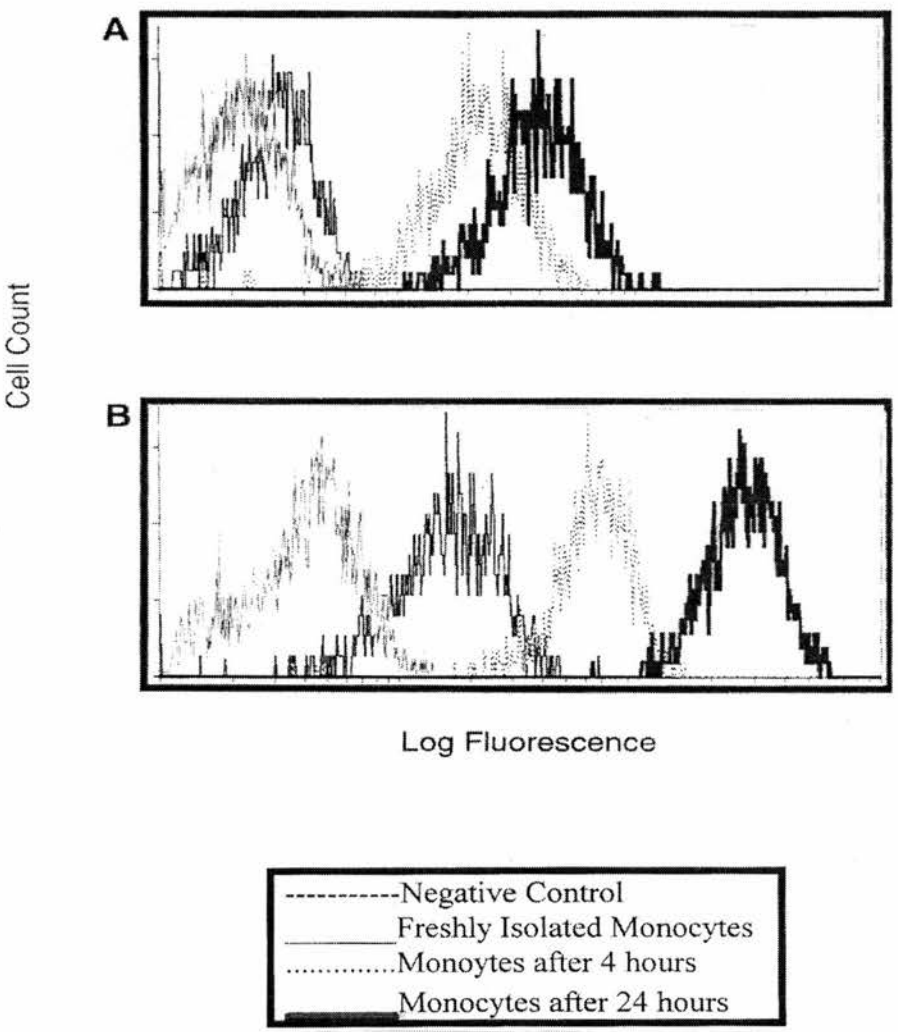


Figure 5.5

In vitro culture of monocytes adherent to tissue culture plastic, resulted in up-regulated expression of CD44, assessed by indirect immunofluorescence and flow cytometry, particulary the variant isoforms v3 (A), and v10 (B).

Pre- incubation with 5A4 (final dilution 1:20 from stock solution, see section 2.2.16 and figure 2.7) significantly reduced TNFα production in culture-derived macrophages but not freshly isolated monocytes.

Experiments with culture-derived macrophages revealed that 5A4 inhibited TNFα release by 47% whereas another CD44 antibody; 7F4 was unable to reduce this TNFα release (figure 5.7). Although both 5A4 and 7F4 recognise epitopes on the N-terminal, ligand-binding region of CD44, the epitope recognised by 7F4 is apparently not involved in the hyaluronan-mediated TNFα release.

The release of TNFα was also observed with macrophages adherent to plastic with exogenous hyaluronan added in solution (figure 5.8) but this was not inhibited with 5A4 (figure 5.9).

5.2.5 Quantitation of monocyte adhesion to immobilised Hyaluronan

To try to determine the molecular basis of observed effects of hyaluronan on monocyte function the adhesion of monocytes to hyaluronan was then investigated. Initially a colorimetric assay was employed using methylene blue (Oliver et al., 1989, see section 2.2.11). However, differences in the numbers of cells adherent to different substrates were masked by cells at the edges of wells remaining adherent throughout the washing procedures. Although there were visible differences in cells remaining in the centre of wells after fixation, once the cells were stained and lysed the optical densities revealed no significant differences between any of the substrates (data not shown). Subsequent assays were therefore assessed by microscopic analysis, 4 random fields were counted in each well, avoiding the edges of the wells.

Initial experiments showed that cells were more adherent to control substrates (plastic and fibronectin) than hyaluronan, and that this adhesion was dependent on the presence of divalent cations (figure 5.10). These observations suggested that the conditions of the assay favoured integrin-mediated adhesion.

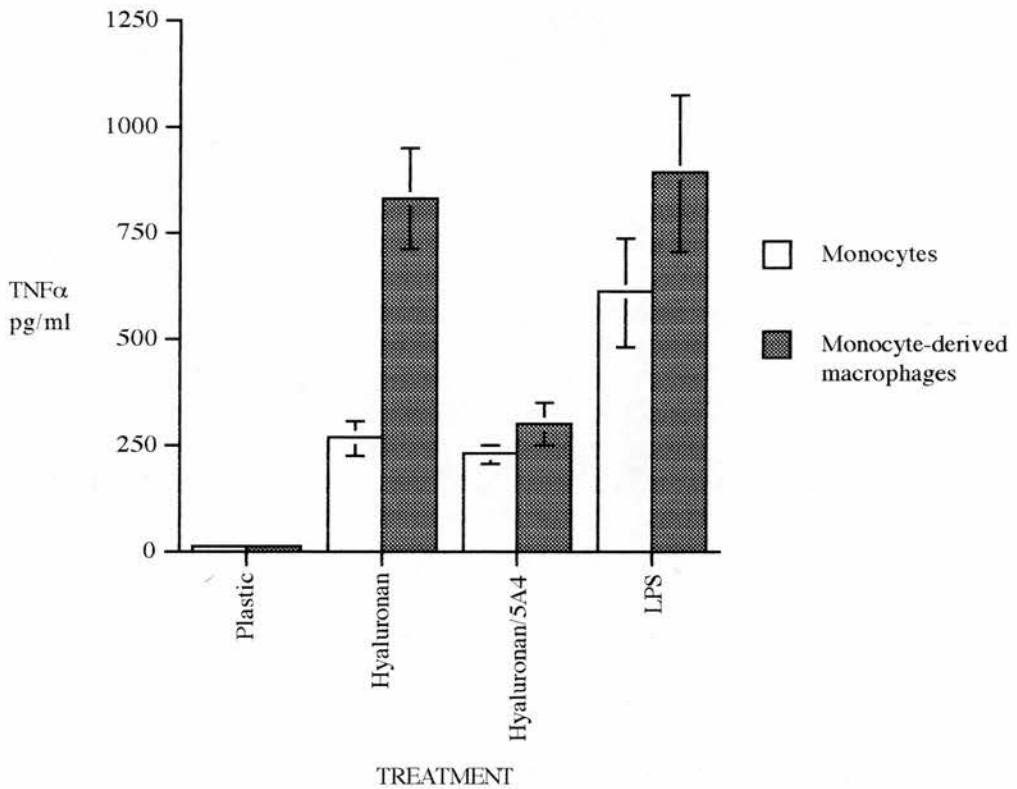


Figure 5.6

A comparison of hyaluronan-mediated TNFα release in monocytes and in vitro-derived macrophages, from the same donor, revealed that macrophages release more TNFα than monocytes, and that this is partially inhibited by the anti-CD44 monoclonal antibody 5A4. Results taken from one representative experiment of 4 performed, mean of triplicate wells ± standard deviation.

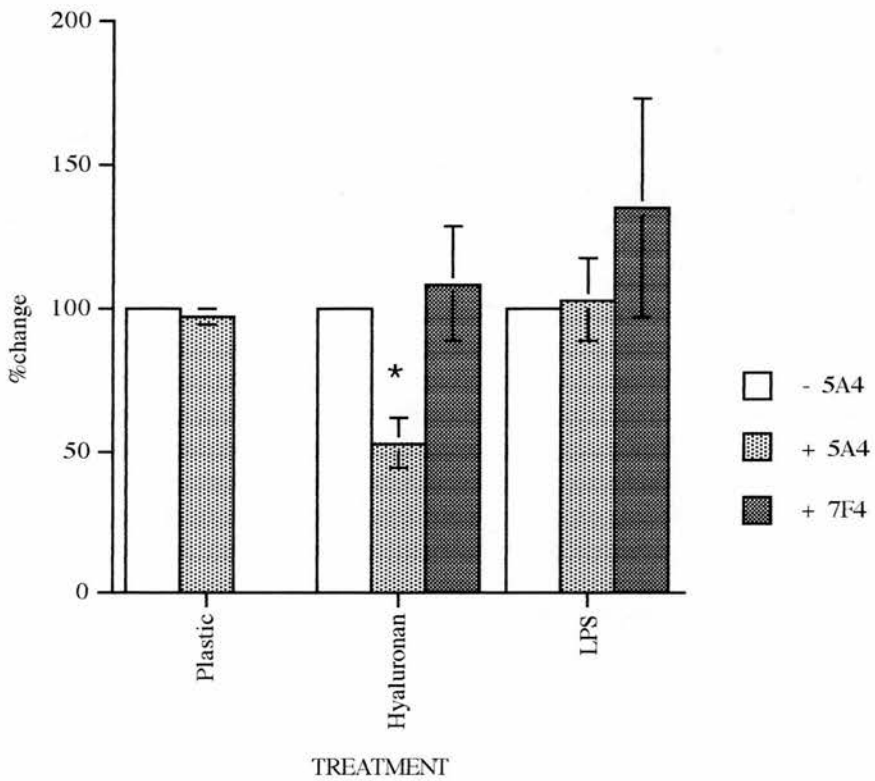


Figure 5.7

Pre-incubation of monocyte-derived macrophages with the anti-CD44 monoclonal antibody 5A4 significantly inhibits the release of TNFα in response to adherence to hyaluronan-coated tissue culture plastic (p=0.01), 7F4 had no significant effect. Results expressed as a percentage of control, n=4.

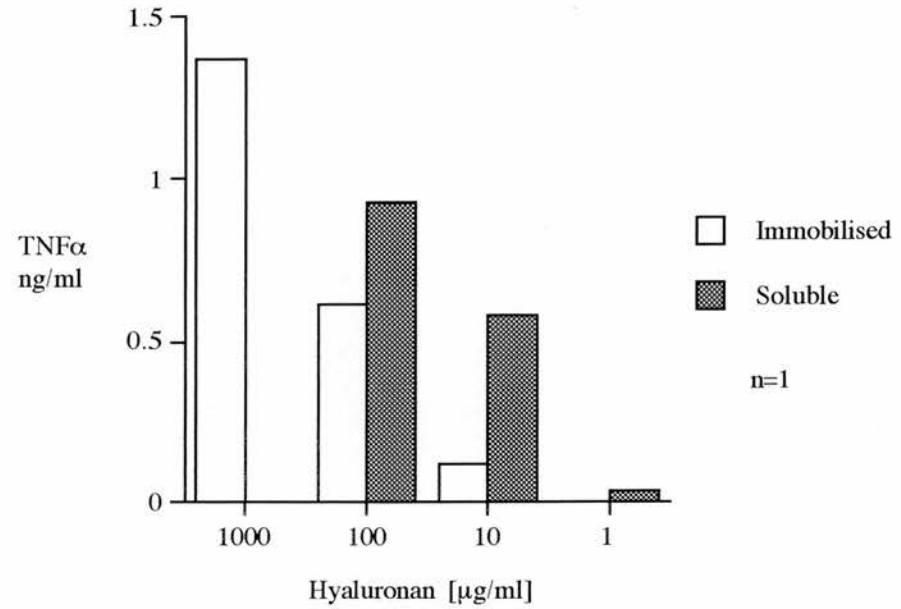


Figure 5.8

Monocyte-derived macrophages release TNFα in response to hyaluronan in a dose dependent manner. Results from one experiment.

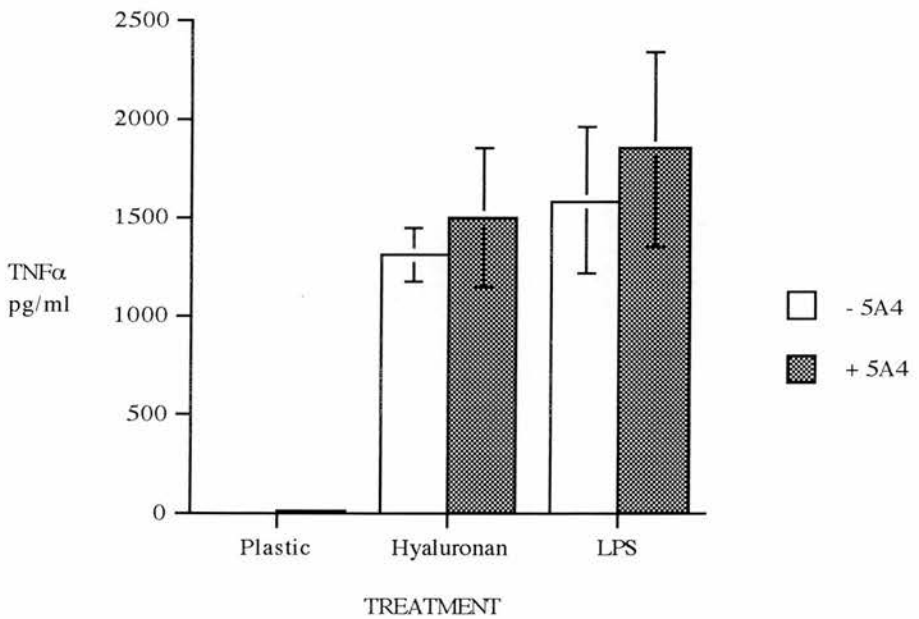


Figure 5.9

Monocyte-derived macrophages release TNFα in response to 100μg/ml soluble hyaluronan which is not inhibited by pre-incubating with the anti-CD44 monoclonal antibody 5A4, n=4.

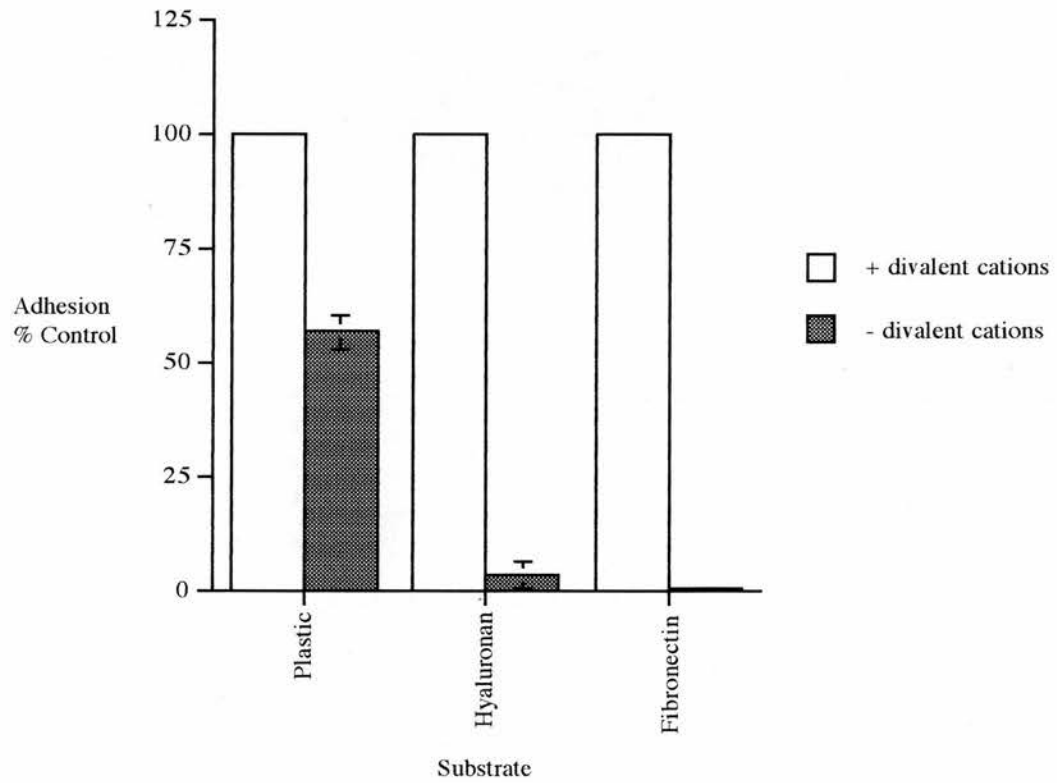


Figure 5.10

Monocytes were allowed to adhere to hyaluronan (1mg/ml) and fibronectin 10μg/ml coated plastic for 30 minutes at 37°C. In the presence of the divalent cations calcium and magnesium, some adherence to hyaluronan and fibronectin was observed. This adhesion was completely inhibited in the absence of calcium and magnesium (n=5).

To attempt to investigate non-integrin mediated adhesion an alternative assay was employed to assess the amount of adhesion to hyaluronan through receptors other than integrins (figure 5.11). To reduce the possibility of integrin-mediated adhesion, the assay temperature was reduced from 37°C to 20°C, and 4°C. In an attempt to overcome cell losses during washing, plates were centrifuged (205 x g for 1 minute) prior to removal of medium. However these modifications also resulted in very low cell numbers remaining adherent to hyaluronan, suggesting that immobilised hyaluronan does not support shear resistant monocyte adhesion.

As monocytes and macrophages release TNFα in response to hyaluronan in solution and hyaluronan did not mediate firm adhesion in these cells, hyaluronan may be providing additional regulatory signals, which give rise to functional effects.

5.2.6 Investigation of the Effects of Hyaluronan on Monocyte Apoptosis

This suggestion raised the possibility that other monocyte functions may be affected by hyaluronan. Since pro-inflammatory cytokine production was induced by hyaluronan, an effect that required gene transcription, one possibility was that hyaluronan might influence cell differentiation, or survival. Monocytes, cultured in the absence of serum *in vitro* readily undergo apoptosis (Mangan et al., 1991). Therefore, in this study, monocytes were cultured in the presence and absence of serum to compare the rates of apoptosis induced in monocytes cultured on tissue culture plastic and on hyaluronan-coated tissue culture plastic.

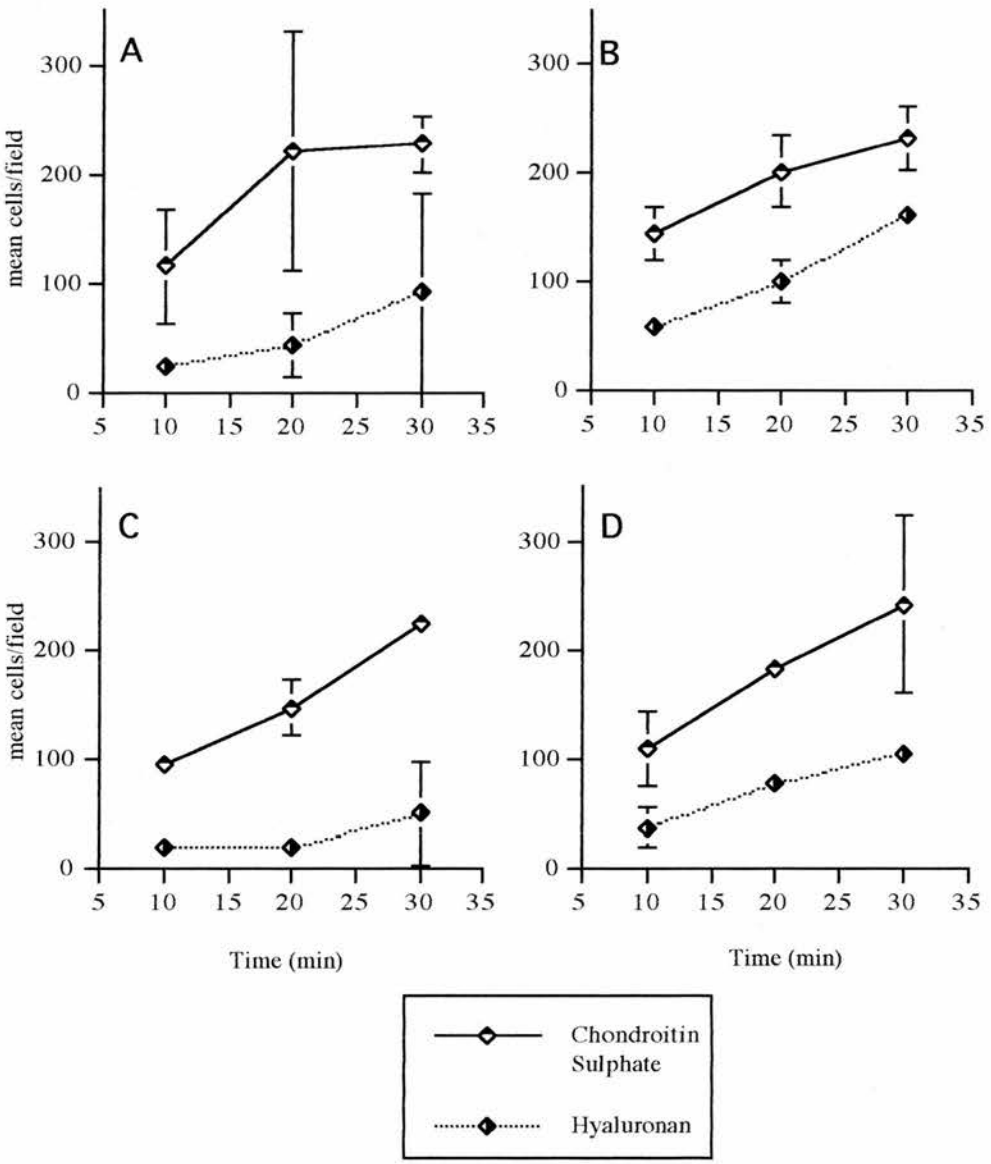


Figure 5.11

Monocytes were allowed to adhere to 96 well plates pre-coated with either 10μg/ml chondroitin sulphate, or 1mg/ml hyaluronan at either 4°C (graphs A and C) or at 22°C (graphs B and D). In addition, plates represented by graphs C and D were centrifuged at 205 x g prior to removing medium, to minimise cell losses, n=2.

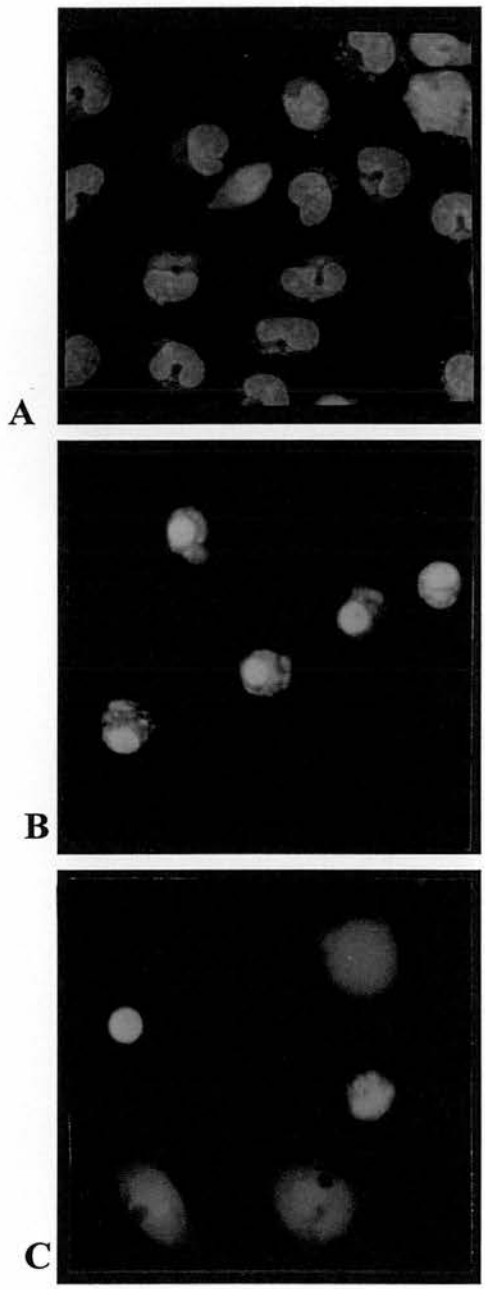


Figure 5.12

Monocyte apoptosis was assessed morphologically through the use of Acridine orange and fluorescence microscopy (chapter 2).
Panel **A** shows typical morphology of monocytes in the presence of serum. These cells are adherent and exhibit the characteristic 'horse-shoe'-shaped nuclei associated with viable cells.
Panel **B** shows monocytes undergoing apoptosis in the absence of serum. During apoptosis chromatin condenses, and apoptotic cells are recognised by the brightly stained, and rounded nuclei.
Panel **C** also shows serum-deprived monocytes, some of which have undergone nuclear extrusion (evanescence), these cells were referred to as 'ghost' cells.

Rates of apoptosis were assessed using morphological criteria illustrated in figure 5.12, and table 5.1 shows the rates of apoptosis observed under these conditions, excluding 'ghost' cells (n=4).

Table 5.1 Effect of Hyaluronan on Monocyte Apoptosis

		No Serum		10% Serum	
Time	**Substrate**	**% Apoptosis**	**% Viable**	**% Apoptosis**	**% Viable**
8 hours	Hyaluronan-coated tissue culture plastic	4.4	81.8	1.1	94.2
	Tissue culture plastic	8.0	72.6	1.2	97.5
20 hours	Hyaluronan-coated tissue culture plastic	5.6	69.5	2.4	91.5
	Tissue culture plastic	3.7	70.2	2.2	96.63
40 hours	Hyaluronan-coated tissue culture plastic	3.4	65.4	4.3	86.5
	Tissue culture plastic	3.1	75.3	2.4	97.4

At 8 hours, serum-deprived monocytes cultured in the presence of hyaluronan showed a lower rate of apoptosis than serum-deprived monocytes cultured on plastic (4.4% compared with 8.0%). When compared statistically this difference did not quite reach significance.

Interestingly, cells on hyaluronan in the presence of serum had higher rates of apoptosis than serum-deprived cells at 40 hours, again this was not statistically significant.

Microscopic examination of cell morphology revealed marked differences in the presence and absence of serum. Monocytes adherent to plastic cultured in the

presence of serum remained round (figure 5.12, A) whereas those cultured without serum became stellate (figure 5.13).

These striking morphological differences were not observed in cells cultured on hyaluronan (not shown).

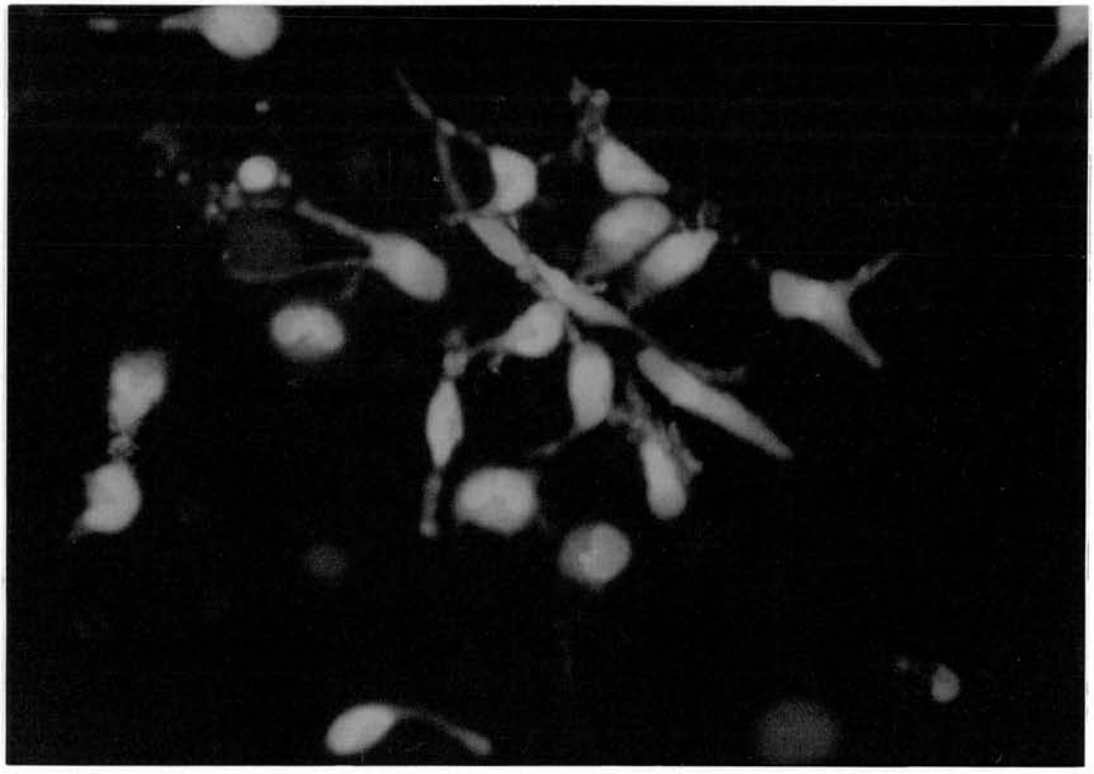


Figure 5.13

Photomicrograph of monocytes cultured on tissue culture plastic, in the absence of serum, after 40 hours (magnification x 400). Cells were visualised using Acridine Orange and fluorescence microscopy.

These cells exhibited a high rate of apoptosis compared with cells cultured in the presence of serum. Interestingly, they displayed a striking stellate appearance, which was not seen in cells cultured in the presence of serum.

5.3 Discussion

In this chapter I have investigated the possible role of hyaluronan in inflammatory processes. My data indicate that hyaluronan is elevated in patients with ARDS consistent with Hallgren et al., (1989) who reported hyaluronan levels between 31 to 1640 ng/ml. Increased levels of hyaluronan in the lung is associated with a variety of situations indicating lung injury, including severity of RDS in premature monkeys (Juul et al., 1994), and bleomycin induced lung injury in rats (Nettlebladt et al., 1989). In addition, the association with veino-lymphatic oedema (Drubraix et al., 1997), and pulmonary oedema in rabbits (Bhattacharya et al., 1989), and rats (Nettlebladt et al., 1989) highlights the osmotic properties of hyaluronan. As hyaluronan molecules in solution behave as highly hydrated random coils and the osmotic pressure increases exponentially with concentration (Comper and Laurent 1978) hyaluronan, like other glycosaminoglycans, provides an excellent physical buffering system. These properties may also contribute to the oedema seen in diseases such as ARDS. However, the physiological significance of the presence of elevated levels of hyaluronan is not clear. A number of physical factors may influence hyaluronan levels. For example increased lymph flow in dogs has previously been shown to reduce lung hyaluronan content (Townsley et al., 1994) and artificial ventilation increases hydrostatic pressure in the microvasculature, and within the interstitium (Hallgren 1989). Increased hydrostatic pressure in ARDS patients, from artificial ventilation, may contribute to the increased hyaluronan in BAL, by mobilising hyaluronan from interstitial tissues, but does not account for the accumulation observed within the interstitium.

The unusual way in which hyaluronan is synthesised may be a contributing factor in the accumulation seen in ARDS. Unlike other macromolecules which are synthesised in intracellular compartments, hyaluronan is secreted from the cell membrane directly into the extracellular space, indicated by the presence of hyaluronan synthase activity co-precipitated with plasma membrane markers (Philipson and Schwartz 1984).

However, the cellular source of hyaluronan in the lung has not been determined. Unlike type I epithelial cells, type II epithelial cells have been shown to produce various matrix proteins (Rannels et al., 1987, Simon et al., 1993, Maniscalco and Campbell 1994) and express CD44 (Kasper et al., 1995), but hyaluronan production in these cells has not been reported, except in foetal type II cells (Sahu et al., 1980, Skinner et al., 1987). Other cells likely to produce hyaluronan include pulmonary endothelial cells, and interstitial lung fibroblasts. Although endothelial cells produce glycosaminoglycan-rich pericellular matrices in response to IL-1 and IFNγ (Montesano et al., 1984), they produce only very small quantities of hyaluronan in culture (Amanuma and Mitsui 1991).

Hyaluronan was prominent in the alveolar interstitium in ARDS lung sections (figure 5.2), a pattern of hyaluronan accumulation which is also observed in bleomycin treated rats (Nettlebladt et al., 1989). This tissue distribution of hyaluronan may implicate the lung fibroblast, as fibroblasts are normally the most numerous cell within the interstitium and synthesise most of the interstitial ECM components (Dunsmore and Rannels 1996). Unstimulated lung fibroblasts express modest levels

of hyaluronan synthase activity, which is increased by TNFα, IL-1 (Sampson et al., 1996), and TGFβ (Westergren-Thorsson et al., 1990).

In normal adult lungs, hyaluronan is restricted to the regions surrounding major blood vessels, bronchi and bronchioles (Underhill et al., 1993). Underhill et al., also noted that hyaluronan is prominent in the interstitium during lung development and decreases on maturation along with an increase in macrophage expression of CD44. As CD44 functions as a receptor for hyaluronan, increased expression in macrophages may provide a mechanism for the clearance of hyaluronan in the developing lung through phagocytosis. This proposed mechanism for hyaluronan clearance is supported by observed intracellular hyaluronan in macrophage vacuoles in bleomycin-induced lung injury (Nettlebladt et al., 1989). The interaction of hyaluronan with macrophages through CD44, may also be an important factor in the pathogenesis of inflammatory disease since CD44 has been implicated in oedema formation associated with collagen-induced murine arthritis (Mikecz et al., 1995).

In the adult lung relatively high CD44 expression on alveolar macrophages has been demonstrated (Viksman et al., 1994). Increased expression of variant isoforms has also been demonstrated in alveolar macrophages (Culty et al., 1994) and may be associated with terminal differentiation of monocytes (Levesque and Haynes 1996). I have demonstrated that the expression of CD44 on monocytes was increased upon maturation *in vitro*, as was the expression of the variant isoforms v3 and v10, thus the use of *in vitro* differentiated monocytes may reflect alveolar macrophage responses better than freshly isolated monocytes.

The myelomonocytic cell lines THP-1 and U937 have been shown to alter variant isoform expression of CD44 in response to cytokines; TNFα preferentially up-regulated v9 expression, IFNγ up-regulated v6, whereas other cytokines (IL-1, IL-4, TGFβ) had little effect (Mackay et al., 1994). It is possible that the environment created by cytokines produced upon adherence, and subsequent maturation of monocytes, influences the expression of CD44 and variant isoforms. Data shown in figures 5.4.1 and 5.4.3 suggest that the extent to which endogenous cytokines affect monocyte CD44 expression *in vitro* may not be great. Monocytes cultured on hyaluronan-coated plastic did not significantly differ in CD44 expression, when compared with monocytes cultured on plastic alone, despite the differences in cytokines in the surrounding medium demonstrated in chapter 4.

Variant CD44 isoform expression is associated with metastasis in tumour cells (Gunthert 1991, Matsumura and Tarin 1992), but the specific functions of variant isoforms in macrophages remain unknown. It is now well established that a cell adhesion mechanism involving such abundant and widely distributed molecules as hyaluronan and CD44 could not be constitutively expressed in an active form if it is to provide any selectivity of interaction, and that specific activation signals would be necessary to elicit function at the appropriate times and sites. Interestingly, resident and elicited peritoneal macrophages have been shown to differ in post-translational modifications in CD44 isoform expression (Camp et al., 1991). Camp et al., found that unlike the CD44 on resident macrophages, the isoform on elicited macrophages was not associated with the cytoskeleton, which may therefore have implications for cell motility. Although the specific consequences of variant isoform expression in

macrophages remains to be determined, increased weak adherence in some cells expressing specific isoforms would be consistent with the suggested role of CD44 in rolling adhesion and motility.

In experiments that aimed to define the nature of CD44 mediated adhesion using static adhesion assays, negligible monocyte adhesion to immobilised hyaluronan was observed. It is therefore probable that CD44 expressed on monocytes is not capable of mediating firm adhesion to hyaluronan. Recent studies have shown that CD44 has been shown to mediate rolling adherence in lymphocytes (DeGrendele et al., 1996, Clarke et al., 1996), similar to the loose tethering mediated by selectins. Clarke et al., observed that the ability of cells to tether decreased with increased shear, which could account for cell detachment during the washing procedures in static adhesion assays. As an alternative approach, monocyte adherence to hyaluronan might have been quantified by using a flow adhesion assay similar to those used by DeGrendele et al., (1996). Despite the difficulty in quantifying monocyte adhesion to hyaluronan, the cytokine release experiments presented in chapter 4 clearly demonstrate significant TNFα release from monocytes on hyaluronan-coated plastic, suggesting that hyaluronan-mediated firm adhesion is not required to induce cytokine release.

Previous studies have shown that freshly isolated monocytes do not bind soluble hyaluronan (Culty et al., 1994, Levesque and Haynes 1996). However, following 8-16 hours *in vitro* culture, binding of hyaluronan-FITC by monocytes was observed by Levesque and Haynes, and by 4 days hyaluronan binding had reached comparable levels to alveolar macrophages (Culty et al., 1994). In both these studies, the levels

of hyaluronan binding coincided with increased surface expression of CD44, suggesting that monocyte maturation, which is associated with up-regulation of CD44 expression, results in altered responses to hyaluronan. Data presented in this thesis indicate that *in vitro* cultured monocyte-derived macrophages release TNFα in response to hyaluronan. In contrast to monocytes, TNFα release could be partially blocked by pre-treatment with the anti-CD44 mAb 5A4. Interestingly the anti-CD44 mAb 7F4 did not inhibit TNFα release despite the increased levels of expression seen in monocytes in culture. Although 5A4 has previously been shown to prevent hyaluronan binding in a murine lymphoma cell line transformed to express CD44, 7F4 did not (Droll et al., 1995). Thus, the inhibition of TNFα release seen with 5A4 implicates the involvement of an epitope which plays a role in hyaluronan binding in macrophages and that the epitope recognised by 7F4, although associated with monocyte maturation, is not involved in hyaluronan binding.

It is possible that antibody-induced cross-linking of CD44, which is expressed at high levels on the macrophage surface, may act to inhibit hyaluronan-induced TNFα release. However, cross-linking of CD44 by immobilised anti-CD44 antibody, has previously been shown to induce TNFα release (Webb et al., 1990) which would argue against this possibility.

An alternative possibility is that increased CD44 expression on macrophages, associated with maturation, leads to increased hyaluronan binding through CD44 (shown by Levesque and Haynes 1996) and so anti-CD44 antibody pre-treatment of macrophages resulted in significant inhibition of hyaluronan-induced TNFα.

Although up-regulated expression of CD44 has previously been linked with increased hyaluronan binding, high expression of CD44 does not necessarily confer hyaluronan-binding activity. In fact recent studies suggest that increased hyaluronan binding through CD44 is associated with decreased CD44 glycosylation (Katoh et al., 1995, Sy et al., 1996). It is possible that increased hyaluronan recognition in culture-derived macrophages reflects a decreased availability of glucose in the culture medium, which could result in reduced CD44 glycosylation. Reduced glucose concentrations have recently been shown to restrict CD44 glycosylation in CHO-K1 cells (Zheng et al., 1997), which induced the recognition of hyaluronan in these cells.

Soluble hyaluronan was also found to induce TNFα release in monocyte-derived macrophages, an effect that was not inhibited by anti CD44 antibody. One possible explanation for the differential effect of CD44 mAb is that immobilised hyaluronan is structurally different to hyaluronan in solution (Laurent and Fraser 1992), eliciting a different type of response. Alternatively, macrophage adhesion to immobilised hyaluronan, may induce receptor cross-linking, an effect which would be inhibited by CD44 antibody treatment.

Although contamination of hyaluronan by LPS remains a possibility for experiments with soluble hyaluronan, Webb et al., (1990) showed that monocytes did not produce TNFα when added to microtitre plates pre-coated with LPS, at concentrations that directly induce TNFα release, suggesting that contaminating endotoxin did not bind to plastic, or was washed off. Furthermore, the possibility that contaminating LPS has effects in experiments using immobilised hyaluronan seems unlikely, particularly

in view of the experiments incorporating polymyxin-B discussed in chapter 4. However, for studies of the effects of soluble hyaluronan it would be important to include polymyxin-B to address this possibility.

The potential pro-inflammatory effects of hyaluronan on monocyte function raised the possibility that hyaluronan may influence other aspects of monocyte behaviour that would influence progression of inflammatory responses, including cell survival. As inflammation is resolved, the number of monocytes in the inflammatory site decreases. It has been shown that in the absence of activation factors, monocytes in culture undergo apoptosis (Mangan et al., 1991) suggesting that, like neutrophils, recruited monocytes may undergo programmed cell death and be cleared from the inflammatory site.

Mangan et al., (1991) also showed that TNFα promoted monocyte survival in the absence of serum. As hyaluronan induced monocytes to release TNFα (chapter 4) it may be expected that monocyte survival could be enhanced by hyaluronan in the absence of serum. However, inhibition of monocyte apoptosis requires the continuous presence of cytokines (Mangan and Wahl 1991), thus any decrease in the rate of apoptosis through hyaluronan-induced TNFα release in this system would be expected to be temporary. The difference in apoptosis rates between serum-deprived monocytes in the presence and absence of hyaluronan was not great. However, it is conceivable that the presence of TNFα may have contributed to this small change. Although not statistically significant a small percentage change could be significant physiologically. Further experiments, perhaps including the use of neutralising antibodies to TNFα, would be necessary to convincingly demonstrate this.

Another possibility is that the ligation of hyaluronan through alternative hyaluronan binding receptors may provide a direct signal for altered cell survival. A number of observations indicate a role for hyaluronan receptors in providing positive and negative signals that regulate cell death. Ligation of CD44 with hyaluronan, or monoclonal antibodies to CD44 has been shown to inhibit T-lymphocyte apoptosis (Ayroldi et al., 1995). In contrast, a recent study has shown that fibroblast apoptosis is induced through CD44 ligation (Henke 1996). These differing responses to CD44 ligation in different cell types remain unexplained.

Interestingly, apoptosis in colon cancer cells and T-lymphocytes has previously been associated with shedding of surface CD44 (Gunthert et al., 1996, Howie et al., 1994), another illustration of the involvement of CD44 in cell survival signals. It would be interesting to observe the survival responses of alveolar macrophages to hyaluronan, as these cells are noted for their longevity. Indeed, although macrophage apoptosis has been demonstrated *in vitro* (Munn et al., 1995, Bingisser et al., 1996), it has not been observed *in vivo*. *In vivo* evidence suggests that macrophages appear to be removed via the lymphatic system (Bellingan et al., 1996), or via the mucociliary escalator (Weibel 1984).

The stellate appearance of monocytes cultured on plastic in the absence of serum is also interesting. Neither monocytes on hyaluronan, nor on fibronectin (data not shown), exhibited this morphology.

Receptors involved in tethering monocytes to different substrates, and the type of adhesion may in turn influence the cell morphology. Initial adherence to plastic is thought to be mediated largely by integrins (Buescher et al., 1985, Rosen and Gordon

1987) and scavenger receptors (Fraser et al., 1993) in a divalent cation dependent manner, consistent with data shown in figure 5.10. However, in the absence of serum factors, initial cell spreading may not be maintained and the monocytes may then de-adhere and retract cytoplasmic extensions, giving rise to a stellate appearance. This could be tested through the use of time lapse photography which has previously been used to observe the formation of stellate fibroblasts (BHK21 cells), induced by elevation of cAMP levels. This response occurs through the outgrowth of processes and is inhibited by the addition of serum (Edwards et al., 1993). Human serum albumin has been shown to inhibit cAMP production in mononuclear cells (Fischer et al., 1992), which may suggest that cells cultured in the absence of serum become stellate through elevated intracellular cAMP levels. Monocyte maturation into macrophages rather than dendritic-like accessory cells during *in vitro* culture may be promoted by serum, which is required to overcome the effects of cAMP (Najar et al., 1990).

Another possibility is that in the absence of serum factors, monocyte production of cytokines such as IL-4 might drive monocytes to assume a stellate morphology characteristic of dendritic cells (Te Velde et al., 1988), this possibility could easily be tested by ELISA. In addition to conferring a dendritic phenotype in monocytes, IL-4 has been shown to enhance monocyte apoptosis (Mangan et al., 1992) which would be consistent with the higher apoptosis rates observed in these stellate monocytes.

The data presented in this chapter indicate that hyaluronan, which has been shown to mediate inflammatory responses in monocytes (chapter 4), also results in macrophage production of TNFα. The partial inhibition of macrophage TNFα release by anti-

CD44 antibody clearly indicates the involvement of CD44 in this response. The increased presence of hyaluronan in BAL from ARDS patients and the extent of accumulation within the interstitium may indicate a role for hyaluronan mediated inflammatory responses in the pathogenesis of this disease.

Chapter 6

General Discussion

The inflammatory response is initiated as a result of tissue injury, caused by various stimuli, and leads to vascular changes, leukocyte infiltration and the production of inflammatory mediators, which act in concert to reduce tissue damage and eliminate the provoking stimuli. Cells of the monocyte-macrophage lineage play a central role in inflammation and in the pathogenesis of inflammatory disease states. In addition to their role in eliminating micro-organisms through effective phagocytic and cytocidal mechanisms, monocytes and macrophages produce a vast array of cytokines, growth factors, and chemokines, including TNFα, IL-1, TGFβ, and IL-8 which affect the responses of other cells, and influence the progression of inflammation. In fact, the induction of the inflammatory response is initiated by cytokines such as TNFα (Tracey and Cerami 1994), without which the repair process breaks down (Marino et al., 1997).

During extravasation into tissues, peripheral blood monocytes undergo a series of adhesions and de-adhesions to other cell types, initially vascular endothelium, cells of the interstitium, and epithelial cells. This process of monocyte extravasation also involves interaction with components of the extracellular matrix (ECM), which are normally present in the basement membranes around vascular endothelium and, in the lung, under alveolar epithelial cells. The pulmonary interstitium, which maintains lung integrity during ventilation, also contains ECM components, such as

collagen, elastin, and proteoglycans (Weibel and Crystal 1991). Basement membrane components include proteoglycans such as heparan sulphate (Sannes et al., 1993, Sivaram et al., 1995), collagen (Madri and Furthmayr 1979), and glycoproteins such as fibronectin (reviewed by Farhardian et al., 1996). Provisional matrices, which are present during wound repair and formed through vascular coagulation, may comprise fibronectin and fibrin (Clark et al., 1982), vitronectin (Grinnel et al., 1992), and hyaluronan (Weigel 1986). Thus, extracellular matrix components are one of the first influences on monocytes recruited to sites of injury as they enter tissues.

The aim of this thesis was to characterise the activation of cells of the monocyte-macrophage lineage in inflammation by examining the relationship between cytokine production and binding to ECM.

Ideally these studies would have used alveolar macrophages recovered from bronchoalveolar lavage from patients and healthy volunteers. Although some experiments presented in chapter 3 used these cells, the culmination of technical difficulties, including the unreliable recovery of reasonable numbers of viable alveolar macrophages and problems with autofluorescence, meant that the use of alveolar macrophages was not practicable. In addition, the frequency of trauma samples decreased significantly at this time, through the introduction of car safety features, improved resuscitation methods and more efficient ambulance response times. As the availability of alveolar macrophages from trauma victims was reduced, the use of alternative models for alveolar macrophages was therefore explored.

The mouse alveolar macrophage cell line MH-S (Mbawuike and Herscowitz 1989), the mouse macrophage cell line RAW 264.7, utilised by Noble et al. (1993), or

murine macrophages derived from bone marrow, or the peritoneal cavity represent possible models. However, in view of species differences, extrapolation of data from studies using murine cells to the situation in human disease is difficult. In addition there may be problems with heterogeneous primary cell populations e.g., peritoneal macrophages. Models for human alveolar macrophages including monocytes and "monocyte-like" cell lines U937 and Mono-Mac 6, present different difficulties. For example, the differentiation of U937 cells to macrophage-like cells involves their stimulation with vitamin D_3, or PMA which activates PKC (Nishizuki 1984), and may make interpretation of results more difficult. Although Mono Mac 6 cells have been reported to represent a more mature monocyte than U937 cells (Zeigler-Heitbrock et al., 1988, Erl et al., 1995), they retain monocyte characteristics, such as expression of myeloperoxidase (Abrink et al., 1994). In addition, myeloid cell lines are inherently different from macrophages, as they are actively dividing cells with altered cell cycle characteristics arising from the original leukaemia, and cell characteristics can alter if they are not maintained properly.

Monocytes differ from macrophages in many respects, including phagocytic ability, expression of surface antigens, and their responses to ECM. Thus the use of monocytes as a model for alveolar macrophages is less than ideal. However, the *in vitro* culture of monocyte-derived macrophages also presents problems when assessing the effects of adherence substrates, as monocytes bind avidly to tissue culture plastic, an activating event which induces the expression and release of cytokines (Kelley et al., 1987, Haskill et al., 1989, Eierman et al., 1989, Kasahara 1991). In order to avoid adherence activation of monocytes counter current

elutriation was used (chapter 2). As elutriated cells did not survive in culture well, discontinuous Percoll density gradients were used for the remainder of the project. Monocyte maturation has been reported to differ in suspension cultures compared with adherent cells on tissue culture plastic (Triglia et al., 1985), which together with difficulty in keeping non-adherent monocytes viable in suspension cultures, limited experiments with *in vitro* differentiated macrophages. However, results presented in chapter 5 were obtained from comparative experiments using suspension cultured monocyte-derived macrophages. The poor viability of suspension cultured monocytes may be predicted when considering that *in vivo*, monocytes survive for less than 72 hours in the circulation (van Furth and Cohn 1968, Volkman 1970, Whitelaw and Batho 1972) after which time they enter tissues and differentiate into macrophages. During extravasation monocytes undergo various types of adherence (chapter 1) which may provide essential survival signals which delay the induction of apoptosis. In adherent cultures, the presence of serum has been shown to prevent apoptosis, however, serum does not improve the viabilities of monocytes in suspension cultures (Mangan 1993).

Although monocyte-derived macrophages may resemble alveolar macrophages in terms of phenotype more closely than monocytes (chapter 3), human peripheral blood monocytes were used throughout the project as relatively large numbers could be obtained comparatively easily. Recent data suggest that large numbers of monocytes are recruited in chronic inflammatory lung disease (Krombach et al., 1996), thus the data presented in this thesis provides valuable insight to the consequences of cell-ECM interactions which are likely to occur during inflammatory responses.

The results presented in this thesis show that hyaluronan, which is a widespread component of the extracellular matrix, and a major component of the provisional matrix, has a pro-inflammatory effect on monocytes and monocyte-derived macrophages. The effects of hyaluronan on monocytes were found to include the production of cytokines, specifically TNFα and IL-1β. Evidence presented in chapter 4 argues strongly against the possibility that the pro-inflammatory effect of hyaluronan was due to contaminating LPS. Furthermore, my data indicated that adhesion to a variety of other ECM components, including fibronectin, vitronectin, collagen I and heparan sulphate, which is structurally related to hyaluronan, did not generate similar effects. Thus the presence of hyaluronan in ECM might exert regulatory effects on progression of the inflammatory response by invoking pro-inflammatory cytokine production.

Receptors involved in the recognition of hyaluronan include RHAMM (the receptor for hyaluronan-mediated motility) and CD44. RHAMM is expressed at low levels on many quiescent cells, including macrophages (Turley 1992), and as the name suggests, is involved in cell motility (Hall et al., 1994). CD44 was first identified as a lymphocyte homing receptor (Goldstein et al., 1989, Stamenkovic et al., 1989), and is expressed on both monocytes and macrophages. One interesting finding in these studies was the difference in responses to hyaluronan between monocytes and monocyte-derived macrophages. In monocytes, firm adhesion to hyaluronan did not occur, and the function blocking anti-CD44 antibody 5A4 did not inhibit hyaluronan-induced TNFα release. This suggests that the mechanism for hyaluronan-induced TNFα release in monocytes does not involve CD44.

Although RHAMM is expressed at low levels on some leukocytes, including stimulated T-, and B-lymphocytes (reviewed by Hall and Turley 1995), and on macrophages (Turley 1992) its expression on monocytes has not been reported. However, the involvement of other hyaluronan receptors has not been precluded. Interestingly, CD54 (ICAM-1) has been shown to be a receptor for hyaluronan in hepatic endothelial cells (McCourt et al., 1994). Although CD54 is expressed on monocytes it remains to be determined if it is involved in monocyte-hyaluronan interactions. The question of which monocyte receptors mediate hyaluronan effects could be addressed by using a panel of monoclonal antibodies to monocyte surface antigens, including CD54. The possibility of modulatory effects of CD54 on monocyte-hyaluronan interactions could also be assessed using soluble CD54.

In contrast to monocytes, the production of TNFα by monocyte-derived macrophages in response to hyaluronan was found to involve CD44. Macrophages have previously been shown to bind hyaluronan through CD44 (Culty 1994, Levesque and Haynes 1996), and results presented in chapter 5 reveal that CD44 expression is up-regulated during *in vitro* culture of monocytes. Although up-regulated expression of CD44 associated with maturation has previously been linked with increased hyaluronan binding, high expression of CD44 does not necessarily confer hyaluronan-binding activity. In fact treatment of a T-cell line with PMA has previously been shown to induce hyaluronan-binding (Lesley et al., 1990), which may implicate the involvement of PKC. More recently, U937 cells stimulated with PMA released TNFα in response to hyaluronan (Boyce et al., 1997), it is therefore possible that PKC activation promotes the binding of hyaluronan through CD44. Although polymyxin-B, (which was used to investigate possible LPS contamination) is known

to inhibit the activation of PKC (Mazzei et al., 1982, Aida Y et al., 1990), results in chapter 4 suggest that PKC was not inhibited by the concentrations used in this study. The possible involvement of a potential PKC phosphorylation site on the CD44 molecule (Camp et al., 1991) involved in hyaluronan-binding or hyaluronan-mediated cytokine production in monocytes or macrophages could be confirmed using specific PKC inhibitors, such as staurosporine.

In addition to effects on cytokine production, data presented in chapter 5 indicate that hyaluronan regulates survival of monocytes cultured in the absence of serum-derived survival signals. Thus, the hyaluronan-rich provisional matrix may provide regulatory signals to monocytes entering a site of inflammation, resulting in increased numbers of inflammatory monocytes present through the inhibition of constitutive apoptosis signals, and also inducing pro-inflammatory cytokine release.

A number of observations indicate a role for hyaluronan receptors in apoptotic mechanisms in different cells. Ligation of CD44 has been shown to inhibit T-lymphocyte apoptosis (Ayroldi et al., 1995), and shedding of CD44 was associated with T-lymphocyte apoptosis (Howie 1994), this implies that CD44 plays an important role in the survival of T-cells. The survival of tumour cells is also affected by CD44 shedding (Gunthert 1996), and certain tumour cells lose their metastatic ability when transfected with a construct which produces soluble CD44 (Yu et al., 1997). Thus, it would appear that CD44 ligation confers survival signals which, in the case of leukocytes, may result in sustaining inflammatory responses. However, CD44 ligation may have differential regulatory effects on survival in different cell types. In fibroblasts, apoptosis is induced through CD44 ligation (Henke 1996), an

effect which may act to further reduce the production of hyaluronan and other ECM components.

Other effects of CD44 on macrophages that might affect the progression of inflammation include the up-regulation of recognition and phagocytosis of apoptotic neutrophils by monocyte-derived macrophages (Hart et al., 1996) a potential anti-inflammatory effect which appears to be independent of hyaluronan binding. In contrast the results presented in this thesis suggest that hyaluronan may have an overall pro-inflammatory effect consistent with the inhibition of macrophage phagocytosis by hyaluronan (Suzuki and Yamaguchi 1993).

The observation that patients with ARDS had elevated levels of hyaluronan in BAL fluid, and prominent staining for hyaluronan in the interstitium (chapter 5), suggests that excessive deposition of hyaluronan in the lung is indicative of disease. In fact elevated levels of hyaluronan in BAL fluid have been associated with several lung disorders: severe asthma (Sahu and Lynn 1978), cystic fibrosis (Sahu 1980), sarcoidosis (Hallgren et al., 1985), and idiopathic pulmonary fibrosis (also known as cryptogenic fibrosing alveolitis) (Bjermer 1989). These diseases have different characteristics and pathologies. Asthma is characterised by widespread airway obstruction due to inflammation-based changes in airway walls and interstitial matrix composition (Hogg 1997). The contribution of several cytokines, including TNFα, have been noted (Barnes et al., 1995). The aetiology of cystic fibrosis is very different from that of asthma; cystic fibrosis is caused by a genetic defect that affects the control of water and iron transport across epithelial cells (Geddes 1995). The

effect on the lung is the opportunistic colonisation by bacteria, resulting in a localised immune response, and inflammation. Sarcoidosis and idiopathic pulmonary fibrosis are diseases with unknown aetiologies, and very different pathologies. Sarcoidosis is characterised by the formation of granulomas in the lung (Studdy 1995). These form through the significant accumulation of macrophages and T-lymphocytes, and cytokines, generated by these cells predominate (James 1997). Progression to a fibrotic state can also occur (Hallgren et al., 1985). Idiopathic pulmonary fibrosis is characterised by a slow and progressive fibrosis caused by fibroblast deposition of matrix and its degradation by neutrophils (Agostini et al., 1997). Alveolar macrophages contribute to idiopathic pulmonary fibrosis with the production of cytokines including TNFα and TGFβ and are primed for IL-8 production (Nakamura et al., 1995). Although the underlying pathology and clinical progression of these diseases are quite different, inflammation is a common feature. In fact increased hyaluronan has also been associated with lung transplant rejection (Riise 1996) which represents another inflammatory state of the lung. Thus inflammation must affect the dynamic nature of ECM in the lung and may therefore affect the deposition or accumulation of hyaluronan (see figure 6.1).

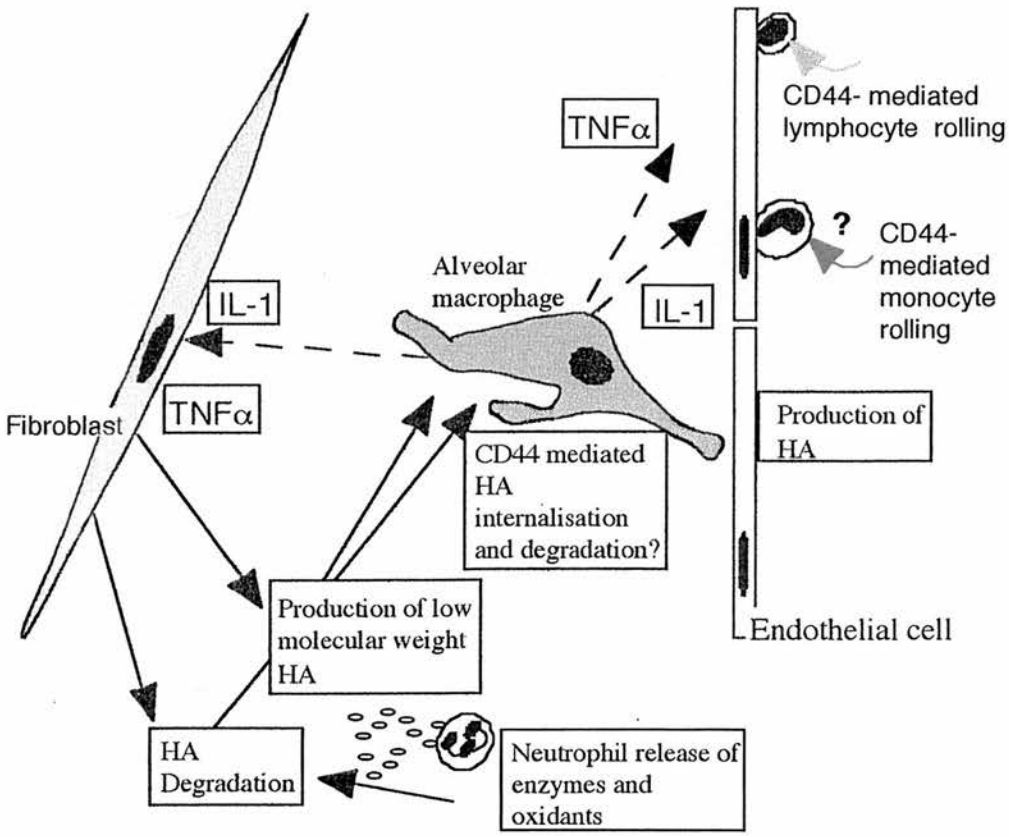


Figure 6.1

Possible mechanism for the potential inflammatory role of hyaluronan within the lung. Macrophage production of pro-inflammatory cytokines, including TNFα and IL-1 results in the production of hyaluronan by endothelial cells. This may facilitate the recruitment of further inflammatory cells through CD44-mediated rolling, and inhibit rates of apoptosis in recruited monocytes.

In addition to hyaluronan degradation by hyaluronidase activity in cytokine-stimulated fibroblasts and neutrophil oxidants, production of low molecular weight hyaluronan is favoured in fibroblasts by TNFα and IL-1. This low molecular weight hyaluronan may further stimulate macrophages, thus prolonging or enhancing the inflammatory response.

The data presented in this thesis suggest that high levels of hyaluronan in the lung are associated with disease. However, high levels of hyaluronan are found in the developing lung (Underhill et al., 1993) and hyaluronan is important in embryogenesis (Fisher and Salrush 1977, Erickson and Perris 1993). During embryonic development hyaluronan may, in part, be responsible for specifying sites of angiogenesis (Banerjee and Toole 1992). Feinberg and Beebe (1983) observed that blood vessels fail to develop in hyaluronan-enriched tissues *in vivo*, suggesting that high concentrations of hyaluronan inhibit angiogenesis, whereas low concentrations do not (West and Kumar 1989). Hyaluronan deposition may thus provide a mechanism for the formation of less well vascularised areas optimised for gas exchange. The presence of high levels of hyaluronan in foetal wound healing is also thought to facilitate healing without scarring (Longaker et al., 1989, Lorenz and Adzick 1993, Estes et al., 1993).

The consequences of high levels of hyaluronan in embryonic development and foetal wound healing would appear to provide an ideal mechanism for wound repair in the adult lung, where the presence of hyaluronan could facilitate scarless healing and prevent excessive angiogenesis. Why then, should high levels of hyaluronan have such a negative association in the mechanism of adult lung repair?

The process of foetal wound healing is regenerative rather than fibrotic, is rarely accompanied by an inflammatory response (Rowlatt 1979, Longaker et al., 1989a), and early embryonic wound healing takes place in the absence of macrophages (Hopkinson-Wooley et al., 1994). In contrast, wound repair in adults often leads to the formation of scar tissue, initiated by ECM components laid down by wound fibroblasts, notably a poorly organised collagen matrix (reviewed by Prockop and

Kivirikko 1995). In cutaneous wound healing this fibrotic response seems appropriate in order to produce a physical barrier quickly from an often unclean environment. However, in the lung, this mechanism of repair is inappropriate and leads to thickening of the interstitium and an impairment of gas exchange interfaces (Bitterman 1992).

The influences of inflammation and macrophages in adults may also provide answers to this question. During inflammation, ECM components are often subject to enzymatic degradation (Wahl and Corcoran 1993, Shapiro 1994), in foetal healing the degradation of hyaluronan by hyaluronidase (Mast 1992) has been shown to result in increased collagen deposition, which is more characteristic of adult wound healing. In an acute inflammatory state such as ARDS degradation of ECM is highly probable, considering the likely presence of neutrophil proteases and matrix metalloproteases (Palmgren et al., 1992, Torii et al., 1997). It is probable that unlike during foetal healing, hyaluronan deposited in an inflammatory environment such as that seen in ARDS is subject to degradation, either through enzymes, or through oxidant effects (Baker et al., 1989, Saari et al., 1993), and that the degraded hyaluronan may have a more inflammatory potential than intact hyaluronan. Indeed, small molecular weight fragments of hyaluronan have been shown to activate murine macrophages (McKee et al., 1996, 1997). In fact, the production of low molecular weight hyaluronan by lung fibroblasts is favoured by the presence of IL-1 and TNFα due to increased synthase activity, and simultaneous increase in hyaluronan degradation (Sampson et al., 1992).

The presence of macrophages in adult lung repair may thus be instrumental in altering the cellular responses to hyaluronan, especially when considering that the

expression of CD44 on macrophages increases during development (Underhill et al., 1993) and in adults, alveolar macrophages express high levels of CD44 (Culty 1994, Viksman et al., 1994). Although the underlying mechanism of repair in adults may thus be similar to those observed in the foetus, altered cellular responses to hyaluronan in adults may result in a very different outcome.

The work presented in this thesis demonstrates the presence of elevated hyaluronan levels in inflammatory lung disease (ARDS) and the potential pro-inflammatory effects of hyaluronan on monocytes and monocyte-derived macrophages, through the production of pro-inflammatory cytokines and the alteration of apoptotic mechanisms. The presence of elevated levels of hyaluronan within the pulmonary inflammatory milieu may therefore alter the relationship between macrophages and the responses of other cells, and may enhance and sustain inflammation contributing to the development of disease.

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